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# THE ROLE OF THE μ-OPIOID RECEPTORS IN THE MECHANISM OF ETHANOL-STIMULATED MESOLIMBIC DOPAMINE RELEASE

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# THE ROLE OF THE μ-OPIOID RECEPTORS IN THE MECHANISM OF ETHANOL-STIMULATED MESOLIMBIC DOPAMINE RELEASE

**by**

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## Dedication

This dissertation is dedicated to my parents, Titus Abiola Job and Yeside Oluwatoyosi Job

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**THE ROLE OF THE μ-OPIOID RECEPTORS IN THE MECHANISM OF ETHANOL-STIMULATED MESOLIMBIC DOPAMINE RELEASE**

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The goal of this dissertation was to investigate the role of μ-opioid receptors in the mechanism of ethanol-stimulated dopamine release in the nucleus accumbens shell (NAcS) of rats. The underlying hypothesis is that blockade of the μ-opioid receptors leads to an attenuation of ethanol-stimulated mesolimbic dopamine release. We prepared ethanol-naïve male Long Evans rats (n = 95) for intravenous (i.v.) drug administration and *in vivo* microdialysis (in awake, freely moving animals), and analyzed our samples using HPLC and GC for dopamine and ethanol detection, respectively. In one set of experiments, we looked at the effects of naltrexone, a non-selective opioid antagonist, on ethanol-stimulated mesolimbic dopamine release. First of all, we checked to see if naltrexone affected basal dopamine levels in the NAcS. Thereafter, we looked for a dose of naltrexone (i.v.) that was effective in suppressing the release of dopamine in the NAcS evoked by morphine (1 mg/kg, i.v.). Subsequently, we checked to see if doses of naltrexone that inhibited morphine-evoked dopamine were also effective

in attenuating dopamine release due to ethanol (1g/kg, 10% w/v, i.v.). To do this, we pretreated rats with naltrexone doses, followed 20 min later by morphine, ethanol or saline (all drugs were administered i.v.). In another set of experiments, we looked at the effect of β-funaltrexamine, a selective μ-opioid antagonist, on ethanol-stimulated dopamine release in the NAcS. Similarly to the previous set of experiments, we looked for a dose of β-funaltrexamine (s.c.) that was effective in suppressing the release of dopamine the NAcS evoked by morphine (1 mg/kg, i.v.), and checked to see if this dose of β-funaltrexamine was also effective in attenuating ethanol-stimulated dopamine release in the NAcS. For the β-funaltrexamine experiments, rats were pretreated with β-funaltrexamine (s.c.) 20-25 h before i.v. infusions of saline, morphine and ethanol.

Morphine increased dopamine release in the NAcS. Naltrexone and β-funaltrexamine significantly attenuated morphine-evoked dopamine release. Also, ethanol increased dopamine release in the NAcS. Naltrexone and β-funaltrexamine, at doses effective in attenuating morphine-evoked dopamine release, suppressed the prolongation, but not the initiation of dopamine release in the NAcS due to ethanol. Naltrexone and β-funaltrexamine did not affect the peak concentration and clearance of ethanol in the brain. The conclusion of this study is that the μ-opioid receptors are involved in a delayed component of ethanol-stimulated dopamine release in the NAcS in ethanol-naïve rats. This is the first study to show that the ethanol-stimulated dopamine response consists of a delayed μ-opioid mechanism.

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# Chapter 1. General Introduction

## ETHANOL ABUSE AND DEPENDENCE

### Consequences of Ethanol Abuse

It is not unusual to see a documentary or an exposition about alcohol abuse and its consequences on society. We have seen messages in the media ranging from 'don't drink and drive' and 'friends don't let friends drive drunk' to 'drink, drive, go to jail' and 'drunk driving is a crime'. Excessive alcohol consumption is the third leading preventable cause of death in the United States (U.S.) (Mokdad et al., 2000). Alcohol is involved in nearly one-third of all traffic-related deaths in the U.S (NHTSA, 2008) (see Table 1.1). Apart from traffic-related fatalities, alcohol has been linked, in some cases, to domestic violence (Brewer and Swahn, 2005). The consequences of alcohol abuse to every individual and to society as a whole cannot be overemphasized.

| Year | Total driving fatalities | Alcohol-impaired driving fatalities |
|---|---|---|
| 2000 | 41,945 | 13,324 (31.8%) |
| 2001 | 42,196 | 13,290 (31.5%) |
| 2002 | 43,005 | 13,472 (31.3%) |
| 2003 | 42,884 | 13,096 (30.5%) |
| 2004 | 42,836 | 13,099 (30.6%) |
| 2005 | 43,510 | 13,582 (31.2%) |
| 2006 | 42,708 | 13,491 (31.6%) |
| 2007 | 41,059 | 12,998 (31.7%) |

**Table 1.1**. The percentage of alcohol-related driving fatalities in the United States from 2000-2007. Adapted from NHTSA Traffic Safety Facts - Research Note (2008). NHTSA = National Highway Traffic Safety Administration.

But what is alcohol? Alcohol, in this case, is ethyl alcohol or ethanol. Ethanol usually refers to grain alcohol, and is a colorless, volatile liquid with a mild odor that is obtained by the

fermentation of sugars. The drinking of alcohol beverages is as old as recorded history, and is of immense socio-cultural and religious significance. Ethanol, which is associated with a good feeling, is one of the most widely used, and abused, recreational drugs. Ethanol abuse in susceptible individuals leads to alcohol dependence, also known as alcoholism.

**Ethanol Dependence**

Ethanol dependence is an example of drug dependence. Drug dependence is a chronic neurological disorder characterized by behaviors including loss of control over drug use, compulsive use of a drug, and intense drug seeking. Drug dependence is associated with drugs that are able to affect the structure and function of the brain. These drugs target certain neuroanatomical structures and neurochemical systems that are also associated with natural reward, mood or pleasure. The target neuronal systems, which are thought to be involved in natural rewards, are hijacked by these addictive drugs and modified to perpetuate compulsive drug use.

Ethanol dependence (alcoholism) is one of the most widespread addictions. Alcoholism is medically classified as a disease. The Journal of the American Medical Association (JAMA) defines alcoholism as a primary chronic disease characterized by impaired control over drinking, preoccupation with the drug alcohol, use of alcohol despite adverse consequences, and distortions in thinking (Morse and Flavin, 1992). Indeed, alcoholism is a serious health concern, and in 2001, the World Health Organization (WHO), through the Office of the Director General, estimated that 140 million people worldwide suffer from some form of

alcoholism (Mayor, 2001). Alcohol abuse and dependence have a huge impact on society, and therefore it is very important to understand the way ethanol interacts with the brain to generate addictive behavior, so that therapeutic interventions can be developed to manage the disease. What is it about ethanol that makes it an addictive drug? It is thought that the addictive effects may be due partly to the reinforcing effects of ethanol.

**Assessment of drug reinforcement**

We mentioned in the previous paragraph that ethanol abuse can lead to ethanol dependence. We concluded with the idea that ethanol is able to do this, in part, because it is a reinforcing drug. It is, therefore, important to briefly talk about drug reinforcement in order to enable us to understand why ethanol should be classified as a reinforcing drug. What is drug reinforcement? In very simple words, drug reinforcement can be defined as an increase in the importance of a drug to an organism such that there is an enhancement of a behavioral response aimed at obtaining the effect of the drug. The reinforcing properties of a drug can be measured using the following operant procedures.

Intracerebral self-stimulation (ICSS)

This is an operant technique that allows self-administration, through performance of a specific task, of a weak electrical current to discrete brain areas via an electrode (Olds and Milner, 1954). The electrical current causes an increase in the activity of target neurons, which in turn may lead to reinforcement. If a particular drug reduces the frequency of self-stimulation (ICSS), the drug may be reinforcing, in which case the subject need not administer more electrical current

(Bozarth et al., 1980). However, a reduction in self-stimulation may be due to locomotor- or motivation-impairing effects of a drug. If a particular drug increases responding for ICSS, it may also imply that the drug stimulates motivation, and may be rewarding. Generally, reinforcing drugs decrease ICSS threshold whereas non-reinforcing drugs either decrease or have no effect on ICSS threshold (Kornetsky et al., 1979).

Drug Self-administration

This is a very powerful tool for determining the reinforcing effect of a drug. In drug self-administration, the animal controls the delivery of a drug via different routes (oral, intravenous, intragastric, subcutaneous, and intracerebral) by pressing a lever in an operant chamber. If a particular drug is readily self-administered, that drug satisfies the criteria for a reinforcing substance.

**Ethanol is a reinforcing drug**

Ethanol affects ICSS. For instance, oral self-administration of low to moderate doses of ethanol increases responding for rewarding brain stimulation (Bain and Kornetsky, 1989). Self-administration of ethanol facilitated ICSS, whereas experimenter-delivered ethanol administration did not (Schaefer and Michael, 1987; Kornetsky et al., 1988; Moolten and Kornetsky, 1990). The effects of ethanol on lateral hypothalamic ICSS are dependent on the duration of time after ethanol administration, with a decrease in reward threshold at $\leq$ 20 min (Lewis and June, 1990)

Ethanol is self-administered through a variety of routes. For example, animal subjects self-administer ethanol intragastrically (Sinden et al., 1983; Waller et al., 1984), intravenously (Smith and Davis, 1974; Sinden and Le Magnen, 1982; Lyness and Smith, 1992; Hyytia et al., 1996; Kuzmin et al., 1999; Grahame and Cunningham, 2002), orally (Grant and Samson, 1985; Samson et al., 1988, 2003; Suzuki et al., 1988; Williams and Woods, 1998; Williams et al., 1998, 2001; Doyon et al., 2004, 2005, 2006) and directly into the ventral tegmental area (VTA) (Gatto et al., 1994; Rodd et al., 2004a, b, 2005). The summary of the above cited literature is that ethanol decreases ICSS threshold, and is self-administered, and therefore fulfils the requirements of a reinforcing drug.

Ethanol reinforcement is thought to be involved in the mechanism of ethanol dependence. Ethanol dependence is thought to progress from an early impulsive stage to a late compulsive stage (Figure 1.1) (Koob and Le Moal, 2005). In the impulsive stage, the drive for ethanol-taking behavior is positive reinforcement. As individuals move to the compulsive stage, the drive transitions to

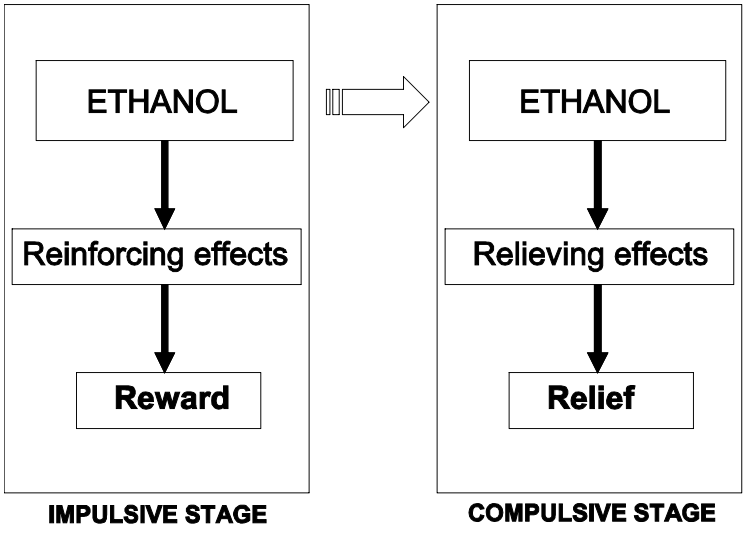



**Figure 1.1**. Proposed stages of alcohol dependence. In the impulsive and compulsive stages, ethanol is taken for its rewarding and negative effect-relieving effects, respectively. Image is adapted from Heilig and Koob, 2007.

negative reinforcement, in which ethanol is taken to achieve the removal of the aversive state (Koob and Le Moal, 2005). In other words, in the early stages of ethanol dependence, ethanol is taken for its rewarding effects, but this soon progresses to a stage in which ethanol is taken to relieve negative effects that occur when ethanol is not used (Heilig and Koob, 2007) (Figure 1.1). Therapeutic interventions targeting ethanol reinforcement may provide benefit in the clinical management of alcoholism.

**Clinical management of ethanol dependence**

We have shown evidence to support the idea of ethanol as a reinforcing drug and described ethanol dependence as a disease. We have talked about how ethanol abuse affects the individual and the society as a whole, and we deem it important to talk about how society is trying to cope with alcoholism. There are many neurochemical mechanisms involved in ethanol dependence, and therefore, there are many possible pharmacological treatments in the clinical management of alcohol dependence (for review, see Heilig and Egli, 2006). The U.S. Food and Drug Administration (FDA) has so far approved only three medications – disulfiram (Antabuse), naltrexone (Revia), and acamprosate (Campral) (Buonopane and Petrakis, 2005). For this dissertation, we will focus on naltrexone. Naltrexone, a non-selective opioid antagonist, when combined with psychosocial treatments, decreases relapse and craving in alcohol dependent patients (O'Malley et al., 1992; Volpicelli et al., 1992; Anton et al., 1999; Chick et al., 2000; Heinala et al., 2001; Kiefer & Mann, 2005; Williams, 2005). In many pre-clinical studies, naltrexone suppressed ethanol reinforcement (Altshuler et al., 1980; Phillips et al., 1997; Bienkowski et al., 1999; Middaugh et al., 1999).

However, some human studies did not find an efficacy for naltrexone (Kranzler et al., 2000; Krystal et al., 2001), probably partly due to poor compliance. Overall, it seems that naltrexone offers marginal benefits. The clinical application of naltrexone in the management of alcohol dependence necessitates a discussion of the endogenous opioid system.

## THE ENDOGENOUS OPIOID SYSTEM

### Discovery of the endogenous opioid peptides and receptors

The discovery of the endogenous opioid system started with the observation that morphine exerts very profound biological effects. Morphine is an opioid alkaloid or opiate (Figure 1.2). The biological effects of opiates, such as morphine, have been known since antiquity, but the knowledge of opiate pharmacology was not established before the 20th century. Opioid comes from the word '*opium*' which is the dried milky juice or latex of the unripe seed capsule of the poppy plant (*Papaver somniferum*). Use of opium poppies for food, analgesia, anesthesia, and ritual purposes dates back to at least the Neolithic Age (9500 BCE). The first known cultivation of opium poppies was by Sumerians in Mesopotamia ~3400 B.C. (Brownstein, 1993). With the widespread use of opium, its addictive effects became apparent. During the Renaissance period, opium was introduced as a tincture (laudanum) in 1527 by the physician

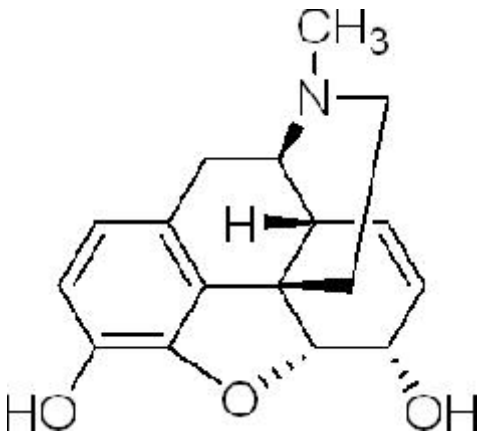



**Figure 1.2**. The chemical structure of morphine ((5α, 6α)-7, 8-didehydro-4, 5-epoxy-17-methylmorphinan-3, 6-diol). Structure reproduced with permission from Sigma-Aldrich.

Paracelsus (1493-1541) for use as a medicinal remedy for a number of indications. Opium is still being used today. Now, opium is known to contain narcotic opiate alkaloids such as morphine, codeine and non-narcotic alkaloids, such as papaverine and noscapine. Morphine was first isolated in 1804 by the German pharmacist Friedrich Wilhelm Adam Sertürner, who named it '*morphium*' (after Morpheus - the Greek god of dreams). Morphine is a very effective analgesic and its use as a pain reliever continues to this day. However, morphine is also a very addictive substance. The discovery of morphine and other opiates and the search for non-addictive opiates has led to extensive characterization of opioid pharmacology.

Based on the unique structural requirements of opiates, the existence of a specific opiate receptor was proposed (Beckett and Casy, 1954a, b). Opiate biological function was observed in guinea pig ileum (Cowie et al., 1970), and subsequently, opiate-binding sites were discovered in the central nervous system (CNS) (Goldstein et al., 1971; Pert and Snyder, 1973a, b; Simon, 1973; Terenius, 1973). In the early 1970s, it was suggested that morphine mimicked certain substances present naturally in the body. The existence of morphine-like endogenous ligands became a subject of interest when it was observed that naloxone, an opiate antagonist, caused aversion in opiate-naïve subjects, and reversed stimulation-induced analgesia. It was hypothesized that the stimulation-induced analgesia occurred as a result of the activation of an endogenous opiate pain modulating system. This idea was supported by the observation that an opiate antagonist (by blocking this endogenous system) reversed this stimulation-induced analgesic effect. In summary, the discovery of the

endogenous opiate binding sites, the intrinsic activity of opiate antagonists in opiate naïve subjects, and the attenuation of non-drug induced analgesia by opiate antagonists, all inspired the idea of the existence of an endogenous system (including endogenous opioid ligands and receptors) (Akil et al., 1976; Buchsbaum et al., 1977).

The existence of endogenous opioids was confirmed by research studies that led to the characterization of enkephalins, the first discovered endogenous opioids (Hughes et al., 1975). Further research led to the discovery of other opioid peptides including β-endorphin and dynorphin (Goldstein, 1976; Goldstein et al., 1979). The endogenous opioids: β-endorphin, enkephalins and dynorphins are formed by enzymatic processing of three precursor molecules, pro-opiomelanocortin (POMC), proenkephalin, and prodynorphin, respectively (Nakanishi et al., 1979; Comb et al., 1982; Kakidani et al., 1982; Noda et al., 1982). The search for other endogenous opioid peptides has led to the discovery of the endomorphins (endomorphin-1 and endomorphin-2) (Hackler et al., 1997; Zadina et al., 1997, 1999). Also, [D-Ala2] deltorphin I has been detected in the brain (Tooyama et al., 1993; Casini et al., 2004).

The concept of opioid receptors arose after the demonstration of opioid-binding sites (see discussion above). Behavioral and neurophysiological findings in the chronic spinal dog distinguished mu (μ), kappa (κ), and sigma (σ) opioid receptors. The mouse *vas deferens* exhibited higher affinity for the enkephalins than for morphine, leading to the proposal for the existence of a distinct enkephalin vs morphine preferring site termed delta (δ) - a receptor that is also

widely distributed in the CNS (Lord et al., 1977; Chang and Cuatrecasas, 1979). The σ receptor does not mediate naloxone-reversible effects and is no longer classified as an opioid receptor. The opioid receptors (μ, δ, and κ) have been isolated, purified and characterized (Simon, 1987a, b; Reisine, 1995). In addition, the human genomic coding regions for these opioid receptors have been identified (Befort et al., 1994; Simonin et al., 1994; Wang et al., 1994; Yasuda et al., 1994).

The differentiation in the binding properties and biological effects of similar opioid ligands has led to the idea of the existence of subtypes of the opioid receptors (Wolozin and Pasternak, 1981; Lutz et al., 1984; Pasternak, 1986; De Costa et al., 1989; Rothman et al., 1989; Jiang et al., 1991; Negri et al., 1991; Wollemann et al., 1993; Hiller et al., 1996). These opioid receptor subtypes include μ1, μ2, δ1, δ2, κ1, κ2, κ3 (Dhawan et al., 1996). Recently, there has even been suggestion of an opioid-peptide insensitive, opioid alkaloid-sensitive μ3-opioid receptor (Stefano et al., 2008). However, the existence of opioid receptor subtypes is controversial because there is no genetic evidence to back up their existence. Subtypes of the opioid receptors may arise from post-translational modification of the receptor (for review, see Wei et al., 2004).

## Opioid receptor pharmacology

### Opioid receptor signaling mechanisms

The three major opioid receptors (μ, δ, and κ) have similar molecular structures and belong to the family of seven transmembrane G-protein coupled receptors. They have been cloned using molecular biological techniques (Evans et al.,

1992; Kieffer et al., 1992; Chen et al., 1993a, b; Meng et al., 1993; Minami et al., 1993; Thompson et al., 1993; Wang et al., 1993; Yasuda et al., 1993; Uhl et al., 1994; Knapp et al., 1995). Activation of the opioid receptors leads to the activation of $G_{i/o}$ protein causing the inhibition of adenylate cyclase and a reduction in cAMP, decreases in calcium channel conductance, and increases in potassium channel conductance (Grudt and Williams, 1995; Law et al., 2000).

Opioid receptor interactions

In addition to the structural and functional characterization of μ-, δ-, and κ-opioid receptors (Simon, 1987a, b; Goldstein and Naidu, 1989), μ- and δ-opioid receptors may dimerize to form physically coupled opioid receptors (Schoffelmeer et al., 1988, 1989, 1990a, b; Heyman et al., 1989a, b; Jiang et al., 1990; Traynor and Elliot, 1993; Jordan and Devi, 1999; George et al., 2000; Gomes et al., 2000; Levac et al., 2002; Wang et al., 2005; Snook et al., 2006; Rutherford et al., 2008) or may interact through non-coupled receptor cross-talk (Sheldon et al., 1989; Malmberg and Yaksh, 1992; Palazzi et al., 1996). The μ- and δ-opioid receptor interactions are illustrated in Figure 1.3. Alt et al. (2002) published findings supporting the hypothesis that μ- and δ-opioid receptors share a common G protein pool, possibly through a close organization of the two receptors and G protein at the

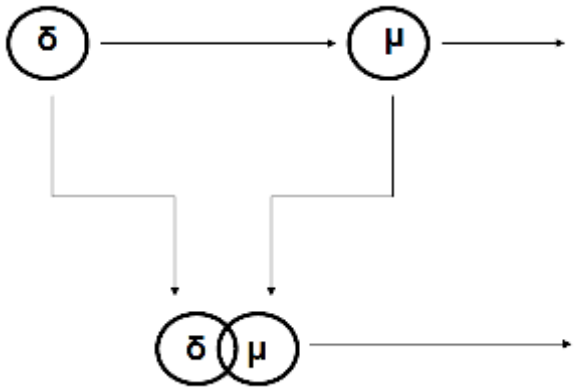



**Figure 1.3**. Opioid receptor interactions. δ- and μ-opioid receptors can interact through non-coupled receptor interaction or through physically coupled δ-μ-opioid receptor heterodimerization.

plasma membrane. Furthermore, Charles et al. (2003) showed that under certain conditions, an interaction occurs between μ- and δ-opioid receptors leading to a change in the functional response to μ-opioid activation.

Some of the evidence regarding the existence of this μ-δ-opioid receptor interaction comes from research looking at the biological activity of [D-Pen2-D-Pen5] enkephalin (DPDPE). DPDPE is a very selective δ-opioid agonist with 1000 fold affinity for δ-opioid compared to μ- and κ-opioid receptors (Raynor et al., 1994; George et al., 2000). However, selective genetic knockout (Sora et al., 1997; Fuchs et al., 1999; Matthes et al., 1998; Hosohata et al., 2000; Scherrer et al., 2004), and selective pharmacological blockade (Fraser et al., 2000), of the μ-opioid receptor resulted in an attenuation of DPDPE-mediated antinociception. It is important to understand that in these μ-opioid receptor knockout mice, δ-opioid receptor density is intact (Kitchen et al., 1997). The evidence suggests that DPDPE is binding at the δ-opioid receptor but also recruiting the μ-opioid receptor.

Selectivity of the endogenous opioid peptides for opioid receptors

The endogenous opioid peptides, as expected, interact with the opioid receptors (Reisine, 1995). However, the endogenous opioid peptides are not very selective, and bind to the various opioid receptors with differing affinities (Hughes et al., 1980; Goldstein and Naidu, 1989). For instance, β-endorphin has affinity and efficacy at the μ- and δ-opioid receptor (Spanagel et al., 1990a; Reisine, 1995), including activity at the μ1-opioid receptor (Houghten et al., 1984). However, β-endorphin also binds to a single protein in the rat striatum that has

affinity for μ- and δ-opioid receptor ligands, but in addition has a higher molecular weight than individual μ- and δ-opioid receptors (Schoffelmeer et al., 1989, 1990a, b). This is proposed to be the physically coupled μ-δ opioid receptor complex (Schoffelmeer et al., 1989, 1990a, b; Bals-Kubik et al., 1990). With regards to μ- and δ-opioid properties, β-endorphin displays affinity in the order μ-δ > δ > μ (George et al., 2000). Other endogenous opioid peptides also bind to various opioid receptors. For instance, the opioid peptides- met- and leu-enkephalins- have high affinity at μ-, δ-, and the μ-δ-opioid receptor complex (George et al., 2000). Enkephalins act at both the μ- and δ-opioid receptor with affinity for δ ≥ μ (Hughes et al., 1980; Raynor et al., 1994; Reisine, 1995). Endomorphin-1 and -2 have a high affinity for the μ-opioid receptor (Monory et al., 2000). Dynorphins have some activity at all opioid receptors (Quirion and Pert, 1981; Zhang et al., 1998), but display a higher selectivity for the κ-opioid receptor (Chavkin et al., 1982; James et al., 1982). The endogenous opioid peptides and their target opioid receptors are summarized in Table 1.2.

**Table 1.2.** The endogenous opioid peptides and their target opioid receptors.

| Opioid peptide | Target receptor(s) |
|---|---|
| β-endorphin | μ, δ, μ-δ |
| Enkephalin | μ, δ, μ-δ |
| Endomorphin | μ |
| Dynorphin | κ |
| D[Ala2]deltorphin I | δ |

**Pharmacology of the μ-opioid antagonists**

After the discovery of the endogenous opioid peptides and opioid receptors, and further advances in opioid chemistry, opioid receptor antagonists were developed. Receptor binding of opioid agonists and antagonists can be differentiated *in vivo* and *in vitro*, with opioid antagonists having 10-1000 fold more potency than agonists (Pert et al., 1973). Like the endogenous opioid peptides, opioid antagonists have differing affinities for μ-, δ-, κ-opioid, and the μ-δ-opioid receptor complex (Raynor et al., 1994; George et al., 2000). In this section, we will focus on μ-opioid antagonists and discuss a few of them that are relevant to this dissertation.

Naltrexone

Naltrexone (Figure 1.4) reversibly blocks all opioid receptors, though it has a 150-fold higher affinity for μ- compared to δ-, and a 4-10-fold higher affinity for μ- compared to κ-opioid receptors (Goldstein and Naidu, 1989; Emmerson et al., 1994; Raynor et al., 1994; Reisine, 1995). The naltrexone opioid-inhibition order is μ > κ >> δ (Takemori and Portoghese, 1984; Goldstein and Naidu, 1989; Emmerson et al., 1994; Peng et al., 2007). Another non-selective opioid antagonist that is well known is naloxone. Naltrexone has

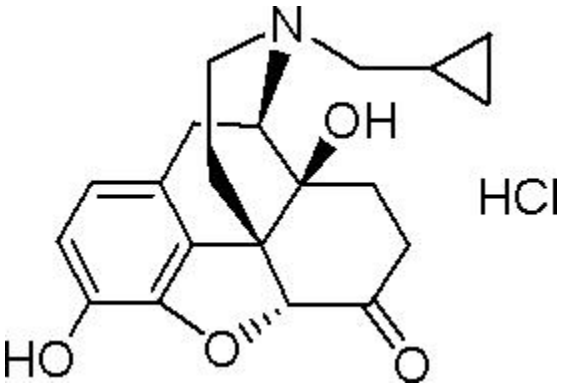



**Figure 1.4**. The chemical structure of the non-selective reversible opioid antagonist: Naltrexone ((5α)-17-(Cyclopropylmethyl)-4, 5-epoxy-3, 14-dihydromorphinan-6-one hydrochloride). Structure reproduced with permission from Sigma-Aldrich.

greater affinity and selectivity than naloxone for the μ-opioid receptor. For instance, compared to naloxone, naltrexone has 2-fold greater selectivity for μ- over the κ-, and a 9-fold greater selectivity for μ- over the δ-opioid receptors (Goldstein and Naidu, 1989; Raynor et al., 1994). Naltrexone is also more potent than naloxone in antagonizing the effects of morphine (Shannon and Holtzman, 1976; Takemori and Portoghese, 1984). Furthermore, naltrexone has a longer duration of action than naloxone (Shannon and Holtzman, 1976).

β-funaltrexamine

β-funaltrexamine (Figure 1.5) is a long-lasting, selective, irreversible μ-opioid antagonist (Ward et al., 1982, 1985). It acts by irreversible alkylation of the μ-opioid receptors (Elliott et al., 1994; Liu-Chen and Phillips, 1987).

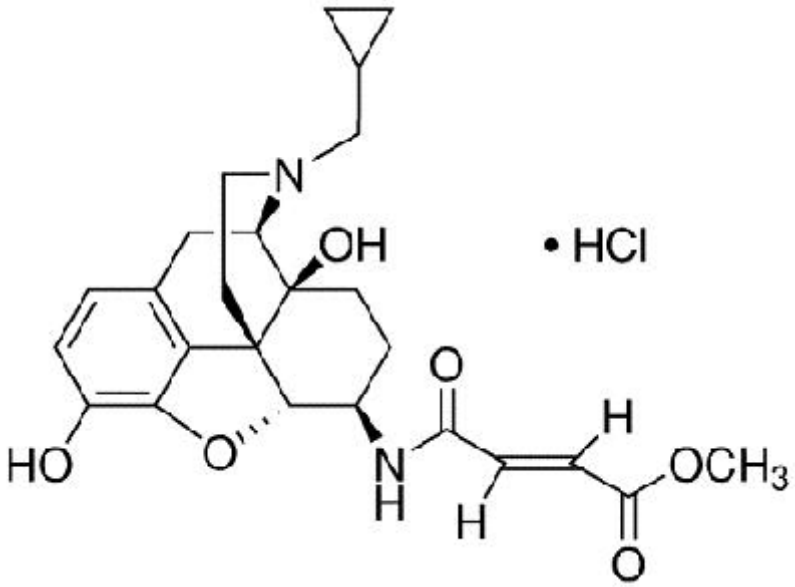



**Figure 1.5**. The chemical structure of the selective irreversible μ-opioid antagonist: β-funaltrexamine ((E)-4-[[5α, 6β)-17-Cyclopropylmethyl)-4, 5-epoxy-3, 14-dih ydroxymorphinan-6-yl] amino]-4-oxo-2-butenoicacid methyl ester hydrochloride). Structure reproduced with permission from Sigma-Aldrich.

β-funaltrexamine is selective for the μ-opioid receptor (Liu-Chen and Phillips, 1987; Liu-Chen et al., 1990, 1991). β-funaltrexamine has a 36-fold selectivity for μ- compared to δ- and a 7-fold selectivity for μ- compared to κ-opioid receptors (Tam and Liu-Chen, 1986).

β-funaltrexamine blocks the effects of the highly selective μ-opioid receptor agonist: [D-Ala2,NMe-Phe4,Gly5-ol]-enkephalin (DAMGO) (Hayes et al., 1985). However, some studies conclude that β-funaltrexamine inhibits the effects of δ-agonists (Dray et al., 1985; Hayes et al., 1985). Further analysis shows that Hayes et al. (1985) used the δ-opioid agonists- [D-Ala2, D-Leu5] enkephalin (DADLE) and [D-Ser2, Leu5] enkephalinyl-Thr6 (DSLET). Goldstein and Naidu (1989) determined that DADLE and DSLET barely distinguish δ- from μ-opioid receptors, and therefore, the conclusion that β-funaltrexamine inhibits the effects of δ-agonist (in this case DADLE and DSLET) needs to be taken with caution. In addition, Dray and colleagues (1985) reported that β-funaltrexamine inhibits the effects of the δ-agonist: DPDPE. However, DPDPE has a very complex pharmacology (as described above in section on opioid receptor interactions), including the possibility of DPDPE acting through δ-opioid and μ-opioid receptor interaction (Figure 1.3). Therefore, it is likely that β-funaltrexamine is inhibiting the actions of DPDPE, by blocking the μ-opioid component of the μ-δ-opioid receptor interaction (see Figure 1.3), and not the δ-opioid receptor. This idea has been confirmed by studies showing that β-funaltrexamine also binds to the μ-δ opioid receptor complex (Rothman et al., 1988, 1991). All the evidence suggests that β-funaltrexamine is selective for the μ-opioid receptor and the μ-opioid

component of a μ-δ-opioid receptor complex. The idea that β-funaltrexamine is active at the δ-opioid receptor needs to be reassessed.

Naloxonazine

Naloxonazine, postulated to be a selective, irreversible μ1-opioid antagonist (Ling et al., 1986), blocked both DAMGO and DPDPE effects (Dray and Nunan, 1984; Dray et al., 1985, 1987). The assumption that naloxonazine may be significantly blocking the δ-opioid receptor (Dray and Nunan, 1984; Dray et al., 1985, 1987), has been predicated on another flawed assumption that DPDPE effects the action in question exclusively via a δ-opioid receptor (see discussion on opioid receptor interactions above). This assumption needs to be carefully considered because naloxonazine does not have any significant affinity for the δ-opioid receptor, and DPDPE has no significant affinity for the μ-opioid receptor (Raynor et al., 1994; George et al., 2000). Naloxonazine has ~200-fold more affinity for μ- compared to δ-opioid receptors (Raynor et al., 1994). It is possible that naloxonazine (similar to β-funaltrexamine) is blocking μ1-opioid receptors involved in an interaction with δ-opioid receptors (see Figure 1.3, sections on opioid receptor interactions and β-funaltrexamine). However, the μ-δ-opioid receptor complex is not sensitive to blockade by naloxonazine (Heyman et al., 1989a), and therefore blockade at μ1-opioid receptors functionally (but not physically) coupled to a δ-opioid receptor, may explain why naloxonazine blocked DPDPE effects (Dray and Nunan, 1984). Additionally, DPDPE may bind with low affinity to the μ1-opioid receptor (Clark et al., 1986). The evidence suggests that naloxonazine may be blocking the μ1-opioid component of the effects of DPDPE (Clark et al., 1986), and not the δ-opioid receptor.

## THE ENDOGENOUS OPIOID SYSTEM PLAYS AN IMPORTANT ROLE IN ETHANOL REINFORCEMENT

We have introduced the endogenous opioid system by discussing the component parts including endogenous opioid peptides and receptors. We mentioned a few important opioid antagonists. Here, we will discuss evidence from pharmacological, genetic, and selective breeding studies, showing that the endogenous opioid system plays a role in ethanol reinforcement.

### The endogenous opioid peptides are reinforcing

Endogenous opioid peptides are reinforcing (for reviews, see Van Ree et al., 1999, 2000; Vaccarino and Kastin, 2001). For example, β-endorphin causes conditioned place preference (Amalric et al., 1987; Bals-Kubik et al., 1990). Enkephalins are self-administered by laboratory animals (Belluzzi and Stein, 1977; Goeders et al., 1984a, b). D-Ala2-Met5-enkephalinamide (a synthetic analog of met-enkephalin) produces facilitation of ICSS (Broekkamp and Phillips, 1979), and conditioned place preference when microinjected into the VTA (Phillips and LePiane, 1982). Endomorphin-1 and -2 produce conditioned place preference when administered into the VTA (Terashvili et al., 2004). In general, β-endorphin, enkephalins, and endomorphins are rewarding, as they are self-administered by laboratory animals whilst the pharmacological profile of dynorphin seems to be different from that of β-endorphin and enkephalins (for reviews, see Van Ree et al., 1999).

Even though dynorphin is generally thought to cause aversive effects, there is some evidence to the contrary. For instance, dynorphin A (1-17) caused conditioned place preference (Iwamoto, 1988, 1989) and dynorphin B, when injected into the VTA, facilitated the self-stimulation rates of the VTA (Singh et al., 1994). There is some evidence that dynorphin binds to all the opioid receptors (μ, δ and κ) with high affinity (Zhang et al., 1998). Dynorphin A is self-administered into the CA3 region of the hippocampus, but this rewarding effect of dynorphin may be due to actions at the μ-opioid receptor (Stevens et al., 1991). Also, endomorphin-2 is reinforcing, but it increases dynorphin levels in the brain (Narita et al., 2001, 2002; Tseng et al., 2002; Wu et al., 2003; Terashvili et al., 2005; Mizoguchi et al., 2006), suggesting that some of the reinforcing effects of endomorphin-2 may even be mediated via dynorphin. Dynorphin may have some reinforcing effects under certain conditions.

In the assessment of drug reinforcement (as discussed in a previous section), μ- and δ-opioid-activating ligands are reinforcing, whereas κ-opioid ligands are not (Van Ree et al., 1999). For instance, intra-VTA administration of DAMGO (selective μ-opioid agonist) and U50488H (selective κ-opioid agonist) produce conditioned place preference and place aversion, respectively (Bals-Kubik et al., 1993). In other studies, the selective μ- and δ-opioid agonists, DAMGO and DPDPE, respectively, were self administered into the VTA (Devine and Wise, 1994). In summary, the endogenous opioid peptides, when acting through μ- and δ-opioid receptors, produce reinforcing effects.

**The endogenous opioid peptides are involved in ethanol intake and reinforcement**

In the previous paragraph, we mentioned that the endogenous opioid peptides may activate μ- and δ-opioid receptors to produce reinforcement. It is thought that ethanol may derive some of its reinforcement through the endogenous opioid peptides (Herz, 1997). It has been suggested that a genetic susceptibility to high alcohol drinking is correlated with plasma β-endorphin activity (Gianoulakis and de Waele, 1994; Gianoulakis, 1996, 2001). Plasma β-endorphin levels were found to be lower in alcoholics compared to normal subjects (Aguirre et al., 1990; Vescovi et al., 1992). In human studies, plasma levels of subjects genetically at high risk for excessive alcohol consumption showed lower basal activity of β-endorphin, and more pronounced release of β-endorphin in response to ethanol (Gianoulakis et al., 1989; Gianoulakis et al., 1996). In rats, β-endorphin interferes with the acquisition and initial maintenance of ethanol preference (Sandi et al., 1989). When administered into the nucleus accumbens (NAc) shell, β-endorphin (1-27), an inhibitor of β-endorphin activity (Spanagel et al., 1991b), inhibits ethanol intake in rats (Resch et al., 2005). The evidence suggests that β-endorphin is important in ethanol intake and in a predisposition to excessive alcohol consumption.

The evidence suggests that enkephalin activity in the brain may affect ethanol intake. Inhibition of aminopeptidase (which leads to an increase in enkephalins) attenuates ethanol intake in rats (Szczepanska et al., 1996b, c). Sandi et al. (1990b) determined that in rats, D-Ala2-Met5-enkephalinamide (a synthetic analog of met-enkephalin) markedly impaired the acquisition of ethanol

preference. Furthermore, in addition to the impairment of the acquisition of ethanol preference, enkephalins also suppress voluntary ethanol intake (Sandi et al., 1990a, c). Enkephalin concentrations in the brain correlate inversely with ethanol intake and the predisposition of different strains of mice to drink ethanol (Blum et al., 1983; Banks and Kastin, 1989). This is buttressed by studies in mice predisposed to high ethanol intake, which show that the administration of enkephalinase inhibitors (which raise brain enkephalin levels) decrease voluntary ethanol intake (Blum et al., 1987). There is data showing that preproenkephalin in the brain is involved in the modulation of neuroadaptative mechanisms associated with the decrease of ethanol intake induced by naltrexone (Oliva and Manzanares, 2007). While the brain enkephalin levels play a role in ethanol intake, there is evidence suggesting that plasma enkephalin levels may not be as important. For example, Vescovi et al. (1992) did not find a difference in plasma met-enkephalin levels between alcoholics and normal subjects.

It is worth mentioning, that apart from endogenous opioid peptides, endogenous opioid alkaloids called tetrahydroisoquinolines (TIQ) can increase ethanol place preference and voluntary ethanol intake (Duncan and Deitrich, 1980). High and low concentrations of tetrahydropapaveroline (THP, a TIQ) suppress and enhance alcohol preference, respectively (Myers and Oblinger, 1977; Blum et al., 1978). However, not all studies have observed ethanol intake following administration of THP (Smith et al., 1980), and others question the relevance of the supporting data (Smith and Amit, 1987; McCoy et al., 2003). The alcohol reinforcing effects of TIQs can be suppressed by naloxone and naltrexone,

suggesting that TIQs activate opioid receptors (Myers and Critcher, 1982; Critcher et al., 1983; Myers, 1989, 1990).

The results obtained from studies looking at the effects of the deletion of opioid peptides on ethanol reinforcement are mixed. For example, β-endorphin wildtypes have a higher preference for ethanol than their knockout littermates (Racz et al., 2008). However, there is conflicting data that indicates that β-endorphin knockouts do not have a lower consumption for ethanol compared to their wildtype littermates (Grisel et al., 1999; Grahame et al., 2000; Hayward et al., 2004). Also, enkephalin wildtype and knockout mice do not show any differences in ethanol consumption and preference (Koenig and Olive, 2002; Hayward et al., 2004). Blednov et al. (2006) showed that prodynorphin knockout mice had reduced ethanol consumption. It is important to take into consideration the compensatory changes due to the deletion of the opioid peptide gene. For instance, both enkephalin and dynorphin knockout mice have significant upregulation of μ- and δ-opioid receptor expression (Brady et al., 1999; Clarke et al., 2003), together with alterations of other endogenous opioid peptide systems.

**The opioid receptors are involved in ethanol intake and reinforcement**

Pharmacological studies

Opioid agonists regulate ethanol intake. Low doses of morphine (1-2.5 mg/kg) increase ethanol intake (Hubbell et al., 1986, 1987, 1988a, b, 1993; Reid and Hunter, 1984; Reid et al., 1986, 1987, 1991; Reid, 1996; Wild and Reid, 1990; Sromberg et al., 1997), whereas high doses of morphine (10-60 mg/kg) decrease ethanol intake (Sinclair et al., 1973; Volpicelli et al., 1991). Also, morphine

suppressed the rebound consumption of ethanol displayed after a period of abstinence to free-choice ethanol in Wistar rats (Sinclair et al., 1973; Sinclair, 1974). Microinfusion of DAMGO into the NAc increased the intake of ethanol (Zhang and Kelley, 2002).

Opioid antagonists suppress ethanol intake and reinforcement. There are numerous data to show that non-selective and selective opioid antagonists suppress ethanol reinforcement (see Tables 1.3, 1.4 and 1.5).

**Table 1.3**. Non-selective opioid antagonists inhibit ethanol intake and reinforcement.

| Non-selective opioid antagonist | Selected references |
|---|---|
| Nalmefene | Hubbell et al., 1991; Mason et al., 1994, 1999; Karhuvaara et al., 2007; Walker and Koob, 2008 |
| Naloxone | Lorens and Sainati, 1978; Marfaing-Jallat et al., 1983; Sinden et al., 1983; Pulvirenti and Kastin, 1988; Sandi et al., 1988; Froehlich et al., 1990; Hyytia and Sinclair, 1993; Hyytia et al., 1999; Overstreet et al., 1999 |
| Naltrexone | Gonzales and Weiss, 1998; Stromberg et al., 1998a, b; Coonfield et al., 2002, 2004; Kiefer et al., 2005; Resch et al., 2005; Higley and Kiefer, 2006; Kuzmin et al., 2008; Gilpin et al., 2008 |

**Table 1.4**. Naltrexone, a non-selective opioid antagonist, inhibits ethanol intake and reinforcement in different mammalian species.

| Species | Selected references |
|---|---|
| Mice | Phillips et al., 1997; Middaugh et al., 1999; Middaugh and Bandy, 2000; Middaugh et al., 2000; Fachin-Sheit et al., 2006; Kamdar et al., 2007 |
| Rats | Franck et al., 1998; Gonzales and Weiss, 1998; Stromberg et al., 1998a, b; Ciccocioppo et al., 2002; Coonfield et al., 2002, 2004; Parkes and Sinclair, 2002; Heyser et al., 2003; Pickering and Liljequist, 2003; Stromberg, 2004; Kiefer et al., 2005; Resch et al., 2005; Burattini et al., 2006; Higley and Kiefer, 2006; Sabino et al., 2006; Jimenez-Gomez and Shahan, 2007; Kuzmin et al., 2008; Gilpin et al., 2008 |
| Non-human primates | Altshuler et al., 1980; Myers et al., 1986; Kornet et al., 1991; Boyle et al., 1998; Williams and Woods, 1998; Williams et al., 1998, 1999 |
| Humans | O'Malley et al., 1992, 2002; Volpicelli et al., 1992; Swift, 1995 |

**Table 1.5**. Effects of selective opioid antagonists on ethanol intake in rats.

| Antagonists | Target | Ethanol intake | Selected references |
|---|---|---|---|
| β-funaltrexamine | μ | decrease | Krishnan-Sarin et al., 1998; Stromberg et al., 1998a |
| CTOP | μ | decrease | HyytÏa , 1993 (i.c.v.); HyytÏa and Kiianmaa, 2001 (i.c.v.) |
| Naloxonazine | μ1 | decrease, no effect | Honkanen et al., 1996; Franck et al., 1998; Mhatre and Holloway, 2003 |
| Naltrindole | δ | decrease | Krishnan-Sarin et al., 1995a; HyytÏa and Kiianmaa, 2001 (i.c.v.) |
| SoRI-9409 | δ | decrease | Nielsen et al., 2008 |
| ICI-174,864 | δ | decrease, no effect | HyytÏa, 1993 (i.c.v.); Krishnan-Sarin et al., 1995a; Franck et al., 1998; |
| Natriben | δ2 | decrease | Krishnan-Sarin et al., 1995b; June et al., 1999 |
| N,N(CH3)2-Dmt-Tic-OH | δ | no effect | Ingman et al., 2003 |
| Nor-BNI | κ | increase | Mitchell et al., 2005 |

CTOP = D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$, i.c.v = intracerebroventricular. SoRI-9409 = 5'-(4-chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-3,14-dihydroxy-4,5α-epoxypyrido-[2',3':6,7]morphinan, ICI 174,864 = allyl$^2$-Tyr-Aib-Aib-Phe-Leu-OH ,

As we mentioned previously, naltrexone, a non-selective opioid antagonist, is FDA approved for the management of ethanol dependence. Pre-clinical and clinical data show that naltrexone can attenuate ethanol drinking behavior, (Tables 1.3 and 1.4). By what mechanism does naltrexone attenuate ethanol intake? Because naltrexone is non-selective, it is important to find out which specific opioid receptor, if any, is involved in its effects. There is evidence that selective μ-opioid antagonists decrease ethanol reinforcement. For instance, β-funaltrexamine attenuates ethanol intake in rats (Krishnan-Sarin et al., 1998; Stromberg et al., 1998a). Also, in rats, naloxonazine, a selective μ1-opioid antagonist, antagonizes some reinforcing effects of ethanol and suppresses ethanol consumption (Honkanen et al., 1996; Mhatre and Holloway, 2003), though Franck et al. (1998) did not show a naloxonazine-mediated suppression of ethanol intake. Furthermore, Honkanen et al. (1996) showed that naloxonazine was insufficient to cause a sustained decrease in alcohol drinking. There is evidence that naloxonazine inhibits ethanol-seeking behavior due to associated drug-related environmental stimuli (Ciccocioppo et al., 2002).

There are also data supporting a role for the δ-opioid receptor in ethanol reinforcement. For instance, naltrindole, a selective δ-opioid receptor antagonist, inhibits ethanol-seeking behavior due to associated drug-related environmental stimuli (Ciccocioppo et al., 2002). In addition, SoRI-9409, a novel δ-opioid receptor antagonist, causes selective and long-lasting reductions of ethanol consumption (Nielsen et al., 2008), though it is important to note that SoRI-9409 also activates the μ-opioid receptor (Wells et al., 2001). Furthermore, there was also a significant reduction in ethanol intake following administration of the δ-

opioid receptor antagonist- ICI-174,864 (Franck et al., 1998). Naltriben, a selective δ2-opioid receptor antagonist, suppressed ethanol self-administration (June et al., 1999). Intracerebroventricular naltrindole suppresses ethanol self-administration in rats (Hyytia and Kiianmaa, 2001). However, Margolis et al. (2008) show that intra-VTA administration of a selective δ-opioid receptor agonist (DPDPE) and antagonist (TIPP-ψ) decrease and increase ethanol intake in low ethanol drinking rats, respectively. Also, N, N (CH3)2-Dmt-Tic-OH, a selective δ-opioid antagonist, does not reduce ethanol intake in alcohol-preferring AA rats (Ingman et al., 2003). The role of the δ-opioid receptors in ethanol intake and reinforcement is not clear. For instance, there is evidence that δ-opioid antagonists may enhance, suppress, or not affect ethanol intake under different conditions.

The effects of opioid antagonists on ethanol reinforcement are centrally mediated, since opioid antagonists that do not cross the blood brain barrier do not suppress ethanol intake (Linseman, 1989). This is supported by data showing that centrally located opioid receptors in the VTA, NAc, amygdala and hippocampus (Heyser et al., 1999; Myers and Robinson, 1999; Hyytia and Kiianmaa, 2001; Foster et al., 2004; June et al., 2004; Bechtholt and Cunningham, 2005; Lasek et al., 2007) have been implicated in ethanol intake and reinforcement.

It is important to mention that it has also been proposed that the effects of opioid antagonists may not be specific for the attenuation of ethanol reinforcement, but may be a general suppression of appetitive and consummatory behavior (Reid

and Hunter, 1984; Koob and Weiss, 1990; Weiss et al., 1990; Schwarz-Stevens et al., 1992; Biggs and Myers, 1998; Williams et al., 1998; Williams and Woods, 1999). Others argue that the opioid antagonists, when administered at lower doses (Froehlich et al., 1990) are specific for ethanol reinforcing effects compared to natural reinforcers. This idea is still controversial.

Genetic studies

Unlike the results from the deletion of opioid peptides, studies looking at opioid receptor deletions are more consistent. Mice without the μ-opioid receptor have decreased ethanol intake (Hall et al., 2001; Becker et al., 2002) and self-administration (Roberts et al., 2000). In addition, selective regional knockdown of the μ-opioid receptor gene through antisense and RNA interference in the NAc and VTA, respectively, led to suppression of ethanol intake (Myers and Robinson, 1999; Lasek et al., 2007). Unfortunately, μ-opioid receptor knockout mice have been shown to have increased proenkephalin gene expression and increased δ-opioid receptor binding (Tien et al., 2007), and the compensatory changes due to the gene deletion may contribute to the decrease in ethanol reinforcement observed. Fortunately, studies utilizing selective pharmacological antagonists also reached the same conclusions with regard to a role for the μ-opioid receptor in ethanol intake and reinforcement. For instance, selective opioid antagonists: β-funaltrexamine (Krishnan-Sarin et al., 1998; Stromberg et al., 1998a) and CTOP (Hyytia and Kiianmaa, 2001) are effective in suppressing ethanol intake in rats. The results from opioid receptor gene deletion studies for the other receptors are unexpected. For example, δ-opioid receptor knockout mice showed an increase in ethanol self-administration (Roberts et al., 2001) and

κ-opioid knockout mice showed decreased oral ethanol self-administration (Kovacs et al., 2005).

Selective breeding studies

One way to look at the impact of a receptor on ethanol reinforcement is to try and determine if the receptor is differentially expressed in high vs low alcohol preferring animal models. Table 1.6. shows some differences between strains bred for high and low ethanol drinking.

**Table 1.6**. Differences in opioid peptides and receptors in the brain between high and low ethanol-preferring rat and mice strains (adapted from Murphy et al., 2002).

| | Differences | Selected references |
|---|---|---|
| β-endorphin | ANA≥AA | Gianoulakis et al., 1992; De Waele and Gianoulakis, 1994 |
| Enkephalin | sP>sNP; P=NP; ANA>AA; FH<WKY; C57BL/6>DBA/2 | Nylander et al., 1994; Cowen et al., 1998; Li et al., 1998; Fadda et al., 1999; Jamensky and Gianoulakis, 1999 |
| μ | AA>ANA; sNP>sP; LAD≥HAD | De Waele et al., 1995; Gong et al., 1997; Soini et al., 1998; Fadda et al., 1999; Marinelli et al., 2000 |
| δ | AA≥ANA; NP>P; DBA/2>C57BL/6 | De Waele et al., 1995; De Waele and Gianoulakis, 1997; Soini et al., 1998; Strother et al., 2001 |

Alcohol-preferring/ alcohol non-preferring = P/NP; HAD/LAD; AA/ANA; sP/sNP; C57BL/6/ DBA/2; FH/WKY (Fuller, 1964; McBride and Li, 1998; Murphy et al., 2002)

We have shown through a wide range of pharmacological, genetic, and selective breeding studies, evidence that the endogenous opioid system is involved in ethanol reinforcement. Of the opioid receptors, the μ-opioid receptor is thought to play a major role in ethanol reinforcement. Indeed, there is evidence implicating a role for the μ-opioid receptor genes in specific brain regions in the modulation of neuroadaptative mechanisms associated with the decrease of ethanol intake induced by naltrexone (Oliva and Manzanares, 2007). This is further reinforced by some pre-clinical (Barr et al., 2007) and clinical data (Oroszi and Goldman, 2004; Ray and Hutchison, 2004; Bart et al., 2005) that describe a correlation between a functional polymorphism of the μ-opioid receptor gene (OPRM1) and vulnerability for ethanol dependence. This polymorphism is also thought to be related to the response to naltrexone in the treatment of alcoholics (Oslin et al., 2003; Anton et al., 2006, 2008; Ray and Hutchison, 2007; Haile et al., 2008; Oroszi et al., 2008; Kim et al., 2009), though not all studies agree (Gelernter et al., 2007; Mitchell et al., 2007; Tidey et al., 2008).

**DOPAMINE IS INVOLVED IN THE MECHANISM OF DRUG REINFORCEMENT**

There is a plethora of evidence suggesting that dopamine plays a role in drug reinforcement., including evidence that reinforcing drugs increase dopamine in the mesolimbic system (for reviews, see Wise and Bozarth, 1985; Leshner and Koob, 1999; Pierce and Kumaresan, 2006). It is important to note that reinforcing drugs also activate non-dopaminergic mechanisms (see Boutrel, 2008). For the purposes of this dissertation, however, we focus on dopamine. We will therefore

discuss dopamine, its role in general drug and ethanol reinforcement, and the mesolimbic dopaminergic system.

**Dopamine as a neurotransmitter**

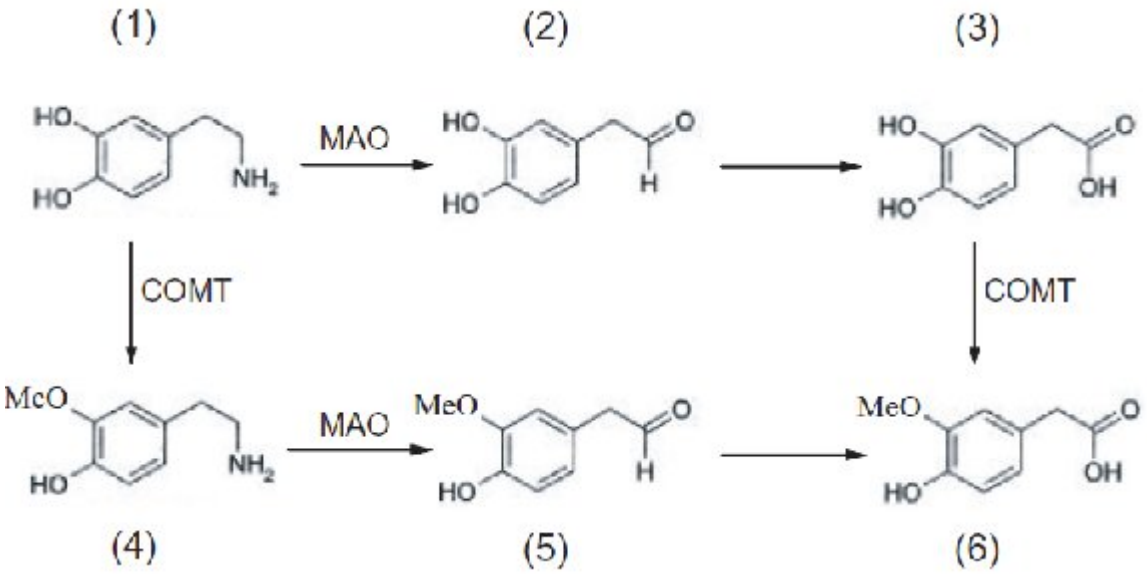
Discovery of dopamine, dopaminergic pathways and dopamine receptors

Dopamine was first synthesized in 1910 by George Barger and James Ewens, but it was not discovered in the brain until the 1950s by Arvid Carlsson and Nils-Âke Hillarp (Montagu, 1957, Carlsson et al., 1958; Bertler and Rosengren, 1959). Dopamine is a neurotransmitter in its own right, but is also a precursor of other neurotransmitters - norepinephrine and epinephrine. It was observed that dopamine was synthesized, stored, and released from neurons in the brain (Portig and Vogt, 1969; Vogt, 1973).

In the 1960s, a fluorescence histochemical method for the detection of catecholamine and indoleamine neurons and their pathways in the brain was developed and used to identify nigrostriatal and mesolimbic dopamine neuronal pathways (Dahlström & Fuxe, 1964; Andén et al., 1965, 1966). Since then other dopaminergic pathways have been identified. The dopaminergic pathways include the mesolimbic, mesocortical, nigrostriatal and tuberoinfundibular pathways. The mesolimbic pathway arises from the VTA and projects to structures in the ventral striatum including the NAc (Weiss and Porrino, 2002). The mesocortical pathway is closely associated with the mesolimbic pathway, and is the pathway from the ventral tegmental area (VTA) to the cortex. The nigrostriatal pathway is the pathway from the substantia nigra to the striatum. The tuberoinfundibular pathway is the pathway from the hypothalamus to the

pituitary gland. The mesolimbic system is thought to be involved in natural reward and drug reinforcement.

Dopamine is synthesized from the amino acid tyrosine by tyrosine hydroxylase into DOPA (dihydroxyphenylalanine), which in turn is converted, via DOPA decarboxylase, into dopamine. Dopamine is metabolized as shown in Figure 1.6.




(1) dopamine
(2) 3, 4-dihydroxyphenylacetaldehyde
(3) 3,4-dihydroxyphenylacetic acid (DOPAC)
(4) 3-methoxy, 4-hydroxyphenylethanamide (3-MT)
(5) 3-methoxy, 4-hydroxyphenylacetaldehyde
(6) 3-methoxy, 4-hydroxyphenylacetic acid (HVA)
MAO = monoamine oxidase, COMT = catechol-O-metyltransferase. Me = methyl.

**Figure 1.6**. Metabolism of dopamine. Figure adapted from images in McCoy et al. (2003).

It was observed that dopamine stimulated adenylyl cyclase (Kebabian et al., 1972). In the search for an anti-psychotic binding site in the brain, it was

discovered that dopamine and haloperidol bound to the same site (Seeman et al., 1976). Not long afterward, subtypes of the dopamine receptor, were discovered, and classified into D1 and D2 based on their pharmacology and coupling to adenylyl cyclase (Kebabian and Calne, 1979). Dopamine receptors, like opioid receptors, belong to the class of G-Protein Coupled Receptors. Activation of the D1 and D2 receptor led to the stimulation and inhibition of adenylyl cyclase, respectively. Since then, molecular biology and genetics enabled the discovery of more dopamine subtypes - D3, D4, and D5 (Strange, 1991). These receptors are classified as either D1-like (D1, D5) or D2-like (D2, D3, and D4) based on their effect on adenylyl cyclase, with the D1 and D2-like dopamine receptors increasing and decreasing adenylyl cyclase, respectively. Dopamine receptors may form homooligomers such as D2-D2 (Lee et al., 2003) and heterooligomers such as D1-D2 (O'Dowd et al., 2005; George and O'Dowd, 2007; Rashid et al., 2007; So et al., 2007). Dopamine receptors can also undergo physical interactions to form heterooligomers with other receptors such as NMDA (Zhang et al., 2009), adenosine A2A (Kamiya et al., 2003; Vidi et al., 2008), serotonin (Lee et al., 2000), cannabinoid CB1 (Kearn et al., 2005) and μ-opioid (Juhasz et al., 2008). The dopamine receptors are found widely in the brain and are well associated with the mesolimbic dopaminergic pathway (Jaber et al., 1996).

**The role of dopamine in drug reinforcement**

Dopamine is thought to play a significant role in drug reinforcement. However, the specific role of dopamine in drug reinforcement is still being debated, and

ideas about dopamine as a direct substrate for reward, and dopamine as a learning signal for reward prediction have been proposed.

Dopamine as a neurochemical correlate of reward

Wise and Bozarth (1987) introduced the psychomotor stimulant theory of addiction. It proposed that dopamine mediates general functions of locomotor activity, behavioral activation, and arousal. This idea is supported by numerous studies (Salamone et al., 1994; Redgrave et al., 1999; Salamone and Correa, 2002). However, there are numerous problems associated with this hypothesis (Salamone et al., 2005). A major limitation of this hypothesis is that it is very general and does not address reward-specific aspects of dopamine activation. Many natural rewards and reinforcing drugs activate mesolimbic dopamine (for reviews, see Wise and Bozarth, 1985; Leshner and Koob, 1999; Pierce and Kumaresan, 2006), and also cause behavioral motivation. It is therefore very tempting to assume that dopamine directly relates to pleasure.

Wise (1978) proposed that dopamine activity is equal to reinforcement, and that dopamine signals are translated to pleasure, with the amount of dopamine being equal to the amount of pleasure. In this regard, by extension, low dopamine or decreased dopamine increase following a stimulus would indicate that the stimulus was not pleasurable or had low hedonic value. However, after 6-hydroxydopamine (6OHDA) depletion of mesolimbic dopamine, animals could still attribute some hedonic value to reinforcers (Berridge et al., 1989; Berridge and Robinson, 1998). Also, non-dopaminergic (dopamine knockout) and hyper-dopaminergic (dopamine transporter knockout mice) states did not affect the

hedonic properties of reinforcers (Cannon and Palmiter, 2003; Peciña et al., 2003; Cannon and Bseikri, 2004; Cagniard et al., 2006). ICSS increases dopamine in the shell and core of the NAc during the acquisition of lever press behavior to receive the ICSS, but after the behavior was established, dopamine release was not continued (Garris et al., 1999). In addition, stress and aversive conditions can also stimulate dopamine release (Abercrombie et al., 1989; Imperato et al., 1992a, b). Taking into account all the above evidence, the idea that dopamine release is directly correlated with reinforcement is doubted.

Dopamine as a prediction signal of reward

More recent ideas propose a role for dopamine in reward prediction and learning. Dopamine may act as a signal that encodes links between a reinforcing agent and associated stimuli (Di Chiara, 1995; Horvitz, 2000; Robinson and Berridge, 2000). For instance, Di Chiara (2002) proposed that a primary function of dopamine is to associate reward produced by the reinforcing substance and the environment stimuli that were present at the time the reward experience occurred (Di Chiara, 2002). The dopamine response that occurs enables a link to be formed between the rewarding effect of the drug and the environment or context in which the drug is taken. However a limitation of this idea is that there is evidence that associative learning can occur in animals with dopamine depletion (Berridge and Robinson, 1998).

Dopamine is thought to be involved in signaling reward prediction errors (Mirenowicz and Schultz, 1994; Schultz, 1997, 1998; Schultz et al., 1997; Hollerman and Schultz, 1998; Wise, 2004). For example, if there is conditioned

stimulus that signals an impending reward, the presentation of the reward has been correctly predicted and there is no prediction error and therefore no increase in dopamine firing pattern. However, if there is an unexpected reward, there is an increase in the firing pattern of dopaminergic neurons. Overall, dopamine integrates the process of learning about the reward, such that environmental stimuli associated with the reward can predict future cases of reward presentation (de la Fuente-Fernandez et al., 2002; Fiorillo et al., 2003; Tobler et al., 2003, 2005). There is some evidence that dopamine neurons adjust their firing after the prediction of reward is learned (Schultz et al., 1997). It has also been suggested that dopamine neurons may not code reward learning or cause any new learning themselves, but may instead be activated as a consequence of learning signals (reflecting learning and prediction) that are generated elsewhere in the brain (Berridge, 2007). Robinson and Berridge (1993) proposed that dopamine attributes incentive salience to stimuli associated with dopamine release, making those events salient and significant. In other words, dopamine may amplify the perception of, and response to, a reward associated stimulus. In this way, dopamine may turn prediction for reward into motivation or incentive, strengthening drug seeking behavior.

**The role of dopamine in the mechanism of ethanol reinforcement**

In previous sections, we classified ethanol as a reinforcing drug. In the last section, we discussed a role for dopamine in the mechanism of general drug reinforcement. It is important to discuss a role for dopamine in the specific mechanism of ethanol reinforcement. Mesolimbic dopamine is thought to be involved in ethanol reinforcement (for review, see Gonzales et al., 2004). Ethanol

increases NAc dopamine during ethanol self-administration (Weiss et al., 1992, 1993; Samson et al., 1993). Just as ethanol administration causes an increase in mesolimbic dopamine, ethanol withdrawal causes a decrease in dopamine release in the NAc (Rossetti et al., 1992; Diana et al., 1993). Systemic dopamine antagonists and agonists alter ethanol self-administration (Pfeffer and Samson, 1988; Hodge et al., 1992; Rassnick et al., 1992; Samson et al., 1992, 1993; Slawecki et al., 1997). Furthermore, administration into the NAc and VTA of a dopamine D2 antagonist and a D2/D3 agonist dose-dependently suppressed ethanol self-administation (Samson and Hodge, 1993). Also, dopamine D1 and D2 antagonists suppress ethanol-seeking behavior (Czachowski et al., 2001; Liu and Weiss, 2002). Intra-NAc administration of fluphenazine, a dopamine receptor antagonist, suppressed conditioned place preference due to intracerebroventricular ethanol administration in rats (Walker and Ettenberg, 2007). Using genetic knockout models, D1 and D2 knockout mice elicited a decrease in ethanol consumption (El-Ghundi et al., 1998; Phillips et al., 1998). Furthermore, dopamine D2 receptor over-expression in the NAc led to a decrease in drinking behavior (Thanos et al., 2001). Low basal activity in the mesolimbic system is thought to be an important factor in determining increased ethanol preference (George et al., 1995).

However, 6-OHDA lesions of the mesolimbic pathway caused variable effects on ethanol self-administration including no change, decrease or increase responding for ethanol in the operant self-administration paradigm in rats (Myers and Melchior, 1975; Brown and Amit, 1977; Quarfordt et al., 1991; Rassnick et al., 1993; Ikemoto et al., 1997; Koistinen et al., 2001). It is suggested that ethanol-

seeking behavior cannot be learned without intact mesolimbic dopamine. However, dopamine may not be as important in ethanol reinforcement once the behavior is learned.

The dopamine response in the NAc may not be a pharmacological effect of ethanol. For example, after repeated ethanol injections, a saline injection also caused NAc dopamine release (Philpot and Kirstein, 1998). Also, in the operant self-administration paradigm, animals anticipating access to ethanol (during waiting period) and during self-administration had an increase in dopamine in the NAc (Weiss et al., 1992, 1993, 1996; Gonzales and Weiss, 1998; Melendez et al., 2002). These data suggest that the conditioned stimuli associated with ethanol are able to promote NAc dopamine release. There is also the idea that it is the novelty of the ethanol experience that causes an increase in mesolimbic dopamine (Nurmi et al., 1996). The dopamine response may also be dependent on the strain of rat used. For instance, there is data showing that in ethanol-preferring (P) rats, compared to Wister rats, there is an increase in dopamine release in the NAc in anticipation of ethanol (Katner et al., 1996).

## THE ENDOGENOUS OPIOID PEPTIDES AND THE μ-OPIOID RECEPTORS INTERACT WITH THE MESOLIMBIC DOPAMINERGIC PATHWAY

We have discussed dopamine and its proposed role in ethanol reinforcement, and we introduced the mesolimbic pathway which is thought to be one of the major reward pathways in the brain. In previous sections, we discussed that the endogenous opioid peptides and the μ-opioid receptors are involved in ethanol reinforcement. Do dopamine and the endogenous opioid systems interact

together to contribute to the mechanism of ethanol reinforcement? It is therefore important to discuss any interactions between the endogenous opioid peptides, the μ-opioid receptors and the mesolimbic dopaminergic pathway.

As mentioned before, the mesolimbic dopaminergic neurons arise from the VTA and project to structures within the ventral striatum, including the NAc (Weiss & Porrino, 2002). The VTA sends dopaminergic and GABAergic projections to the NAc (Van Bockstaele and Pickel, 1995). The NAc is subdivided into core and shell regions (Meredith, 1999; Zahm, 1999). The NAc shell (NAcS) sends GABAergic projections to the VTA (Walaas and Fonnum, 1980; Heimer et al., 1991; Kalivas et al., 1993). VTA dopaminergic neurons are influenced by afferents expressing a variety of neurotransmitters and neuromodulators (for review, see Kalivas, 1993). Subsets of mesolimbic dopaminergic neurons co-localize and co-release the highly selective δ-opioid peptide [D-Ala2] deltorphin I (Dupin et al., 1991; Tooyama et al., 1993; Casini et al., 2004), and [D-Ala2] deltorphin I binding sites have been found in the NAc (Gouarderes et al., 1993; Renda et al., 1993). More research needs to be done to determine the interaction between [D-Ala2] deltorphin I and the mesolimbic dopaminergic system. Interactions with the mesolimbic dopaminergic pathway have been established for some of the other endogenous opioid peptides listed in Table 1.2.

**Neuroanatomical interactions**

Localization of endogenous opioid peptides

The endogenous opioid peptides are located in neurons that interact with the mesolimbic dopaminergic pathway. The opioid precursors (POMC,

proenkephalin, and prodynorphin), opioid peptides (β-endorphin, enkephalin, dynorphin and endomorphin) and opioid receptors have unique anatomical distributions throughout the CNS (Watson et al., 1982a; Akil et al., 1984; Martin-Schild et al., 1999). In the brain, POMC is synthesized in the arcuate nucleus of the hypothalamus and in the nucleus tractus solitarius (Bloom et al., 1978; Finley et al., 1981a), with widespread projections from these sites throughout the brain. β-endorphin containing fibers from the arcuate nucleus of the mediobasal hypothalamus project to the limbic structures such as the NAc (Khachaturian et al., 1984). There is no evidence that β-endorphin containing fibers from the arcuate nucleus of the mediobasal hypothalamus project to the VTA (Khachaturian and Watson, 1982), and β-endorphin immunoreactivity is very sparse in the VTA (Khachaturian and Watson, 1982; Khachaturian et al., 1984). Interestingly, apart from the arcuate nucleus and the nucleus tractus solitarius, low levels of POMC mRNA (previously undetected) have been found in the VTA and NAc (Leriche et al., 2007) and amygdala (Civelli et al., 1982), suggesting that β-endorphin can be synthesized in these regions also. Also, there is a convergence of β-endorphin and enkephalin terminal fibers in the NAc and central nucleus of the amygdala (Finley et al., 1981a, b), suggesting that the opioid peptides may interact with each other. Apart from β-endorphin, POMC is also processed to other endorphins (α- and γ-endorphins), adrenocorticotrophic hormone and β- and γ-melanocyte stimulating hormone. In the arcuate nucleus of the hypothalamus, β-endorphin can be co-localized in neurons with GABAergic (Collin et al., 2003; Hentges et al., 2004), glutamatergic (Collin et al., 2003; Kiss et al., 2005) and cholinergic (Meister et al., 2006; Maolood and Meister, 2008) phenotypes. These neurons project to extra-hypothalamic sites. For example,

some β-endorphin-containing GABAergic neurons are found in the central nucleus of the amygdala (Oertel et al., 1983). Presently, the phenotype(s) of β-endorphin neurons projecting to the NAc are unknown.

Proenkephalin gene encodes a protein which is processed to four copies of met-enkephalin, one copy of leu-enkephalin, and one copy each of two extended met-enkephalin sequences (met-enkephalin-arg-gly-leu and met-enkephalin-arg-phe) (Gubler et al., 1982). Enkephalin containing neurons are found widely distributed throughout the brain and comprise local circuits and long projecting neurons. Enkephalin containing neurons are found in dopaminergic pathways in the substantia nigra, and the dorsal and ventral striatum (Sar et al., 1978; Johnson et al., 1980; Fallon and Leslie, 1986). Proenkephalin mRNA is found in the NAc (Bloch et al., 1986; Harlan et al., 1987; Curran and Watson, 1995). Also, there is evidence of enkephalin synthesizing cells in the VTA (Finley et al., 1981b; Harlan et al., 1987). There are moderate levels of enkephalin immunoreactivity in the VTA (Sar et al., 1978; Johnson et al., 1980; Wamsley et al., 1980a, b; Khachaturian et al., 1983a, b; Garzón and Pickel, 2002), whereas there are significant levels of enkephalin in the NAc (Wamsley et al., 1980a, b; Khachaturian et al., 1983a, b). In addition there are enkephalinergic neurons in structures associated with the mesolimbic pathway such as the amygdala, hippocampus, raphe nuclei amongst others (Khachaturian et al., 1983a, b). Enkephalins are co-localized in some GABAergic neurons (Kalivas et al., 1993; Sesack and Pickel, 1995). Enkephalin-containing neurons project from structures including the NAcS and ventral pallidum to the VTA (Kalivas et al., 1993). However, it is important to add that enkephalin is contained in only about 3% of

the neurons projecting from the NAcS to the VTA (Kalivas et al., 1993; Lu et al., 1998). In the VTA, 50-60% of the enkephalin containing terminals directly form synapses with the dopaminergic neurons, while the rest formed synapses with non-dopaminergic neurons (Sesack and Pickel, 1992).

Prodynorphin gives rise to α-neoendorphin, β-neoendorphin, dynorphin A, dynorphin A (1-8), dynorphin B, and leumorphin (Civelli et al., 1985). Dynorphin immunoreactivity is found widely distributed throughout the brain (Ghazarossian et al., 1980; Goldstein and Ghazarossian, 1980; Hollt et al., 1980; Gramsch et al., 1982; Khachaturian et al., 1982; Watson et al., 1982a, b; Cone et al., 1983). The hypothalamus contains a high density of dynorphin-immunoreactive cell bodies (Khachaturian et al., 1982; Vincent et al., 1982; Roth et al., 1983; Weber and Barchas, 1983; Fallon et al., 1985). There are also dynorphin immunoreactive cell bodies in structures associated with the mesolimbic pathway such as the central nucleus of the amygdala (Weber and Barchas, 1983; Fallon et al., 1985) and hippocampal formation (Khachaturian et al., 1982). There are high densities of dynorphin immunoreactive fibers and terminals in regions such as the substantia nigra, hypothalamus, hippocampal formation, ventral pallidum (Khachaturian et al., 1982; Vincent et al., 1982; Weber and Barchas, 1983; Fallon et al., 1985). Preprodynorphin mRNA is found in GABAergic medium spiny neurons in the NAc, suggesting that many dynorphin producing neurons are found resident in the NAc (Mansour et al., 1994b; Curran and Watson, 1995; Furuta et al., 2002).This is confirmed by observations showing that the NAc contains moderate densities of dynorphin immunoreactivity (Khachaturian et al., 1982; Vincent et al., 1982; Weber and Barchas, 1983; Fallon et al., 1985). There

is moderate dynorphin immunoreactivity in the VTA (Pickel et al., 1993; Fallon et al., 1985). Within the VTA, approximately 22%, 17% and 60% of dynorphin immunoreactive terminals form synapses with dopaminergic, non-dopaminergic and astrocytic targets, respectively (Pickel et al., 1993). As mentioned before, the NAcS sends projections to the VTA (Heimer et al., 1991; Kalivas et al., 1993; Zahm and Heimer, 1993; Lu et al., 1998; Usuda et al., 1998; Zahm et al., 1999, 2001). Unlike for enkephalin (see discussion above), almost all neurons projecting from the NAcS to the VTA contain dynorphin (Zhou et al., 2003). Also, dynorphin afferents from the amygdala and hypothalamus project to the VTA (Fallon et al., 1985). With regard to the hypothalamus, dynorphin is contained almost all hypothalamic orexinergic neurons (Chou et al., 2001) which are of a glutamatergic phenotype, and which form synapses with dopaminergic and non-dopaminergic cells (GABA) in the VTA (Balcita-Pedicino and Sesack, 2007).

Cell bodies of endomorphin-1 and endomorphin-2- containing neurons are found in the hypothalamus (Martin-Schild et al., 1999; Zadina et al., 1999; Pierce and Wessendorf, 2000; Greenwell et al., 2002, 2007), especially in the periventricular nucleus, between the dorsomedial and ventromedial hypothalamus and between the ventromedial and arcuate nuclei of the hypothalamus (Greenwell et al., 2002). From the hypothalamus, endomorphin containing neurons project to the VTA (Pierce and Wessendorf, 2000; Greenwell et al., 2002) and to the NAc (Schreff et al., 1998; Martin-Schild et al., 1999; Whitten et al., 2001). Endomorphin immunoreactive fibers are also seen in structures associated with the mesolimbic pathway such as the raphe nucleus, laterodorsal tegmental nucleus, and the amygdala (Pierce and Wessendorf, 2000).

Localization of the μ-opioid receptors

Opioid receptors are widely distributed in the CNS and are found in the mesolimbic pathway (Quirion et al., 1983; Mansour et al., 1988, 1993; 1994a; 1995a, b, c, d). The different opioid receptors have distinct distributions (Mansour et al., 1987; Tempel and Zukin, 1987). μ-opioid receptors are found in the striatum, cortex, hippocampus, amygdala, raphe nuclei, and thalamic nuclei, amongst others (Mansour et al., 1987, 1994a, c; Tempel and Zukin, 1987). μ-opioid receptors have moderate density in the VTA (Mansour et al., 1987, 1995a, b, c; German et al., 1993; Moriwaki et al., 1996; Garzón and Pickel, 2001, 2002). Within the VTA, most μ-opioid receptors are found on non-dopaminergic neurons (though some dopaminergic neurons express μ-opioid receptors) (Dilts and Kalivas, 1989; Garzón and Pickel, 2001). There is a significant density of μ-opioid receptors in the NAc (Quirion et al., 1983; Moriwaki et al., 1996; Svingos et al., 1996). The μ-opioid receptor is predominantly located extrasynaptically on dendrites and spines of the GABAergic medium spiny neurons of the NAc, or their targets (Svingos et al., 1996, 1997). There are very few μ-opioid receptors on dopaminergic terminals in the NAc (Unterwald et al., 1989).

We have discussed literature showing that μ-opioid receptors are predominantly located on non-dopaminergic neurons in the mesolimbic pathway. Also, we have talked about the neuroanatomical interaction between β-endorphin, enkephalin, dynorphin, endomorphin, the μ-opioid receptor and the mesolimbic pathway. A summarized illustration of the mesolimbic dopaminergic pathway and associated endogenous opioid system is shown in Figure. 1.7.

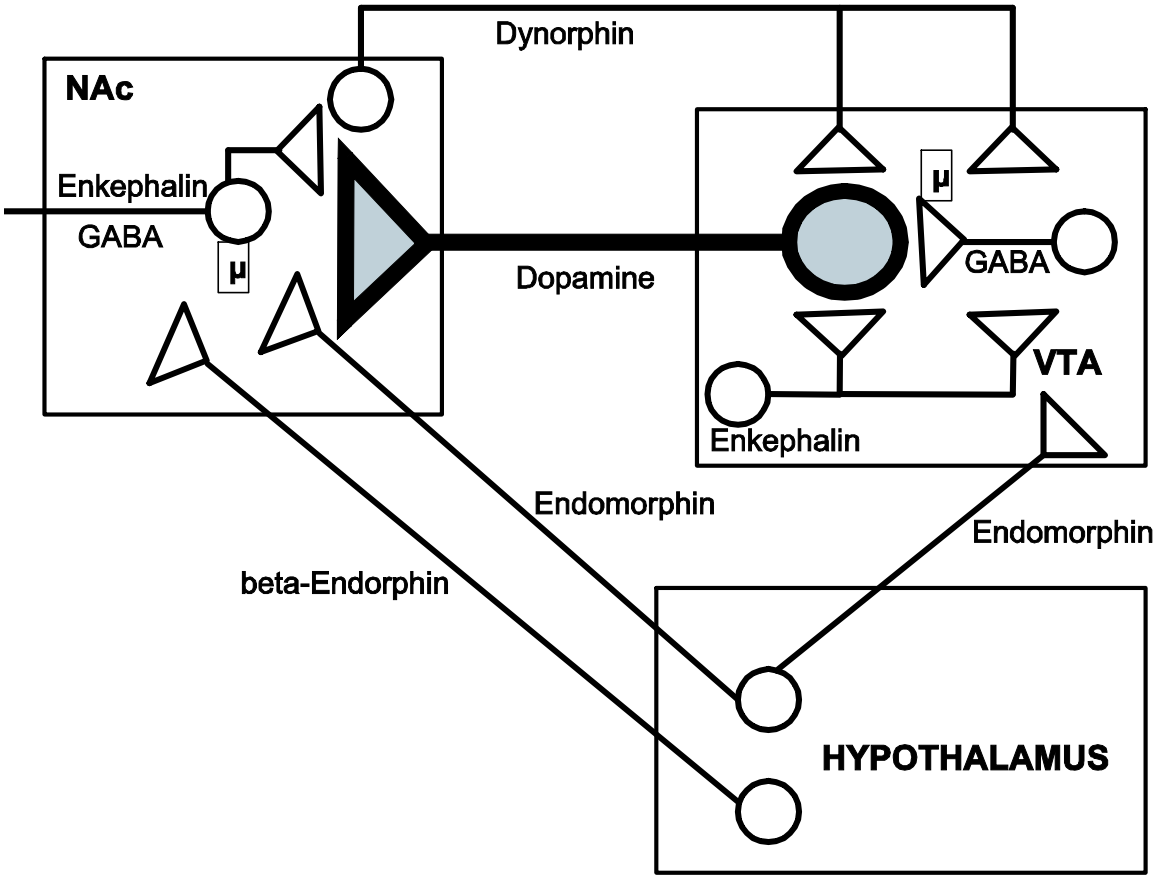



**Figure 1.7**. Hypothetical neuroanatomical interactions between endogenous opioid peptides, the μ-opioid receptors and the mesolimbic dopaminergic pathway. VTA = ventral tegmental area, NAc = nucleus accumbens. Author's concept of cited literature.

### Neuropharmacological interactions

β-endorphin, enkephalin, and endomorphin increase mesolimbic dopamine release

With evidence of neuroanatomical interactions between opioid peptides and receptors and the mesolimbic dopaminergic pathway (see Figure 1.7), there is a high probability that endogenous opioid peptides regulate mesolimbic dopamine. This indeed is the case (see Van Ree et al., 1999). The regulation of VTA

dopamine neurons by opioids is not direct. For instance, VTA dopaminergic neurons do not respond to direct application of enkephalin (Johnson and North, 1992), though not all studies agree (Ford et al., 2006). For example, while Johnson and North (1992) did not find any responses by VTA dopamine neurons to enkephalin, Ford et al. (2006) found that enkephalin directly inhibits the firing rates of about 40% of the VTA dopaminergic neurons that project to the NAc.

β-endorphin, enkephalin, and endomorphin, which all have efficacy at μ-opioid receptors, increase mesolimbic dopamine release. For example, β-endorphin increases mesolimbic dopaminergic neuronal activity (Stinus et al., 1980), and dopamine activity and release in the NAc (Iyenger et al., 1989; Spanagel et al., 1990a, 1991a, b). Also, administration of β-endorphin onto VTA dopamine cells *in vitro* increased dopamine neuron firing (Trulson and Arasteh, 1985). In addition, locally administered β-endorphin increases dopamine release in the NAc (Iyenger et al., 1989). Intracerebroventricularly administered β-endorphin activates the NAc and other associated limbic regions such as the lateral septal nucleus, the amygdalo-hippocampal transition area and the hippocampal formation (Ableitner and Schulz, 1992). Interestingly, data from Ableitner and Schulz (1992) suggests that the NAc, but not the VTA, is activated by intracerebroventricularly administered β-endorphin. This is buttressed by data showing that β-endorphin innervation of the VTA is very negligible compared to the NAc (Khachaturian and Watson, 1982; Khachaturian et al., 1984).

Enkephalins increase dopamine in the NAc (Cador et al., 1989). Intra-VTA administration of thiorphan, an enkephalinase inhibitor, increases dopamine

release in the NAc, suggesting that mesolimbic dopamine is regulated by an endogenous opioid tone and enkephalin contributes significantly to this. This also shows that endogenous enkephalins activate the dopaminergic activity in the VTA (Kalivas and Richardson-Carlson, 1986; Dauge et al., 1992). Intra-VTA administration of endomorphin-1 increases dopamine in the NAc (Terashvili et al., 2008). Intracerebroventricular administration of endomorphin-2 increases dopamine in the shell of the NAc (Huang et al., 2004). Also, endomorphins-1 and -2, when administered locally, increase dopamine in the NAc (Okutsu et al., 2006; Aono et al., 2008; Saigusa et al., 2008).

These findings suggest that there is an endogenous opioid-mediated modulation of dopamine release. We have just discussed that endogenous opioid peptides can increase mesolimbic dopamine. We also show that the endogenous peptides (β-endorphin, enkephalin, endomorphin) that can increase mesolimbic dopamine release have some efficacy at the μ-opioid receptors. It is important, therefore, to discuss the μ-opioid receptors and how they affect mesolimbic dopamine release when activated. An important question to ask is this: Does activation of the μ-opioid receptor lead to an increase in mesolimbic dopamine?

Activation of μ-opioid receptors increases mesolimbic dopamine release

Activation of μ-opioid receptors increases mesolimbic dopamine release. Morphine, which has high affinity and efficacy at the μ-opioid receptor (Raynor et al., 1994), increases mesolimbic dopamine activity and release (Di Chiara and Imperato, 1988b; Leone et al., 1991; Pothos et al., 1991; Rada et al., 1991; Wood and Rao, 1991; Pontieri et al., 1995; Borg and Taylor, 1997; Piepponen et

al., 1999; Hamilton et al., 2000; Melis et al., 2000). This morphine-evoked increase in dopamine release is due predominantly to activation of the μ-opioid receptor. This is supported by evidence showing that morphine-evoked increases in mesolimbic dopamine release were abolished in μ-opioid receptor, and not δ-opioid receptor, knockout mice (Chefer et al., 2003). Furthermore, blockade of the δ-opioid receptors did not attenuate dopamine release in the NAc due to systemically administered morphine (Borg and Taylor, 1997).

Studies with other selective μ-opioid agonists reinforce the observation that activation of μ-opioid receptors increases mesolimbic dopamine activity and release. For example, DAMGO, a highly selective μ-opioid agonist (Emmerson et al., 1994), when administered intracerebroventricularly, caused an increase in dopamine levels in the NAc (Spanagel et al., 1990b), and this effect is blocked by the selective μ-opioid receptor antagonist: D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$ (CTOP). Activation of the μ-opioid receptors in the VTA leads to an increase in dopamine release in the NAc. For instance, administration of DAMGO into the VTA caused an increase in dopaminergic neuron firing activity (Noel and Gratton, 1995) and dopamine release in the NAc (Spanagel et al., 1992; Devine et al., 1993b). Furthermore, systemic administration of naloxonazine, a μ1-opioid antagonist, suppressed the increase in dopamine metabolism in the NAc due to intra-VTA DAMGO (Latimer et al., 1987). Activation of μ-opioid receptors in numerous neuroanatomical structures associated with the mesolimbic dopaminergic pathway can lead to an increase in dopamine release in the NAc (see Table 1.7).

**Table 1.7**. Neuroanatomical structures in which activation of the μ-opioid receptor leads to increases in dopamine in the nucleus accumbens.

| Neuroanatomical structure(s) | Selected reference(s) |
|---|---|
| Globus pallidus | Anagnostakis and Spyraki, 1994 |
| Mediobasal thalamus | Klitenick and Kalivas, 1994 |
| Nucleus accumbens | Yoshida et al., 1999; Hirose et al., 2005; Okutsu et al., 2006 |
| Pedunculopontine tegmental nucleus | Klitenick and Kalivas, 1994 |
| Raphe nucleus | Klitenick and Wirtshafter, 1995 |
| Ventral tegmental area | Spanagel et al., 1992; Devine et al., 1993b |

However, some studies show an increase in NAc dopamine levels with VTA microinjection of μ-opioid receptor antagonists (Devine et al. 1993c) suggesting an intrinsic non-opioid effect of CTOP on neurons (Chieng et al., 1996) or perhaps a more complex VTA circuitry. μ-opioid receptor agonists are thought to increase NAc dopamine by hyperpolarizing (and thus inhibiting) inhibitory GABAergic interneurons/afferents in the VTA (Johnson and North, 1992).

It is still controversial whether local μ-opioid receptor activation in the NAc causes an increase in NAc dopamine release. However, the evidence seems to suggest that μ-opioid receptor activation in the NAc has both a dopaminergic and non-dopaminergic component. For instance, even though some studies have shown that μ-opioid receptor activation in the NAc did not increase NAc

dopamine activity (Longoni et al., 1991; Pentney and Gratton, 1991; Spanagel et al., 1992), others show that μ-opioid receptor activation in the NAc caused an increase in NAc dopamine release (Yoshida et al., 1999; Hirose et al., 2005; Okutsu et al., 2006; Aono et al., 2008; Saisusa et al., 2008). Though it is not well understood, one possible explanation for the dopamine-dependent and dopamine-independent effects of intra-NAc μ-opioid receptors can be done by taking the heterogeneity of the NAc into consideration. In this light, it can be said that there is a percentage of NAc μ-opioid receptors that when activated will lead to an increase in dopamine release in the NAc. The differences in between dopamine-responsive and non-responsive μ-opioid receptors could be due to the region of the NAc in which these receptors are expressed. The regional differences in μ-opioid receptor-driven dopamine response is supported by immunohistochemical data showing 14% more μ-opioid receptor in the shell than in the core of the NAc (Pickel et al., 2004), and pharmacological data showing that local administration of DAMGO increased, decreased or had no effect on NAc dopamine release in the core, shell, and core/shell overlap zones, respectively (Hipolito et al., 2008).

Mesolimbic dopamine can also be increased by activation of μ-opioid receptors that interact with δ-opioid receptors. For example, DPDPE, administered intracerebroventricularly, caused an increase in dopamine levels in the NAc (Spanagel et al., 1990a; Manzanares et al., 1993), and this effect is blocked by the selective δ-opioid receptor antagonists: ICI 174,864 and naltrindole. Administration of DPDPE into the raphe nucleus also led to an increase in dopamine release in the NAc (Klitenick and Wirtshafter, 1995). Additionally, intra-

VTA administration of DPDPE caused an increase in NAc dopamine release (Devine et al., 1993a, b), and this increase was attenuated by naltrindole, a selective δ-opioid receptor antagonist. This suggests that in the VTA, for instance, δ-opioid receptors are involved in mesolimbic dopamine release. However, on closer examination of the data, the concentrations (in the VTA) of DPDPE were 100-1000 fold higher than the concentrations of DAMGO (selective μ-opioid receptor agonist) required to cause this NAc dopamine increase (Devine et al., 1993b). An interpretation is that in the VTA, the δ-opioid receptors are simply less sensitive than the μ-opioid receptors in the mechanism of mesolimbic dopamine release. However, there is *in vitro* data that shows that VTA neurons are insensitive to DPDPE (Johnson and North, 1992) but not to DAMGO. The increase in mesolimbic dopamine release due to DPDPE can be accounted for by the idea of the δ-opioid recruitment of μ-opioid receptor mechanisms. In this idea, DPDPE may occupy δ-opioid receptors that are involved in some form of interaction with the μ-opioid receptors to lead to an increase in dopamine (see Figure 1.3, section on opioid receptor interactions).

Locally applied DPDPE increases dopamine release in the NAc. This is also thought to be through the δ-opioid receptor (Pentney and Gratton, 1991; Manzanares et al., 1993; Suzuki et al., 1997). However, the increase in NAc dopamine through direct δ-opioid receptor activation is still very controversial. For example, systemic administration of non-peptidic selective δ-opioid receptor agonists:BW373U86 ((±)-[1(S*),2α,5β]-4-[[2,5-Dimethyl-4-(2-propenyl)-1-piperazinyl](3-hydroxyphenyl)methyl]-N,N-diethylbenzamide dihydrobromide) and SNC 80 ((+)-4-[(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-

methoxybenzyl]-N,N-diethylbenzamide), failed to modify extracellular dopamine in the NAc (Longoni et al., 1998). Hirose et al. (2005) determined that a combined activation of both δ- and μ-opioid receptors in the NAc is required to cause an opioid-mediated increase in extracellular dopamine in the NAc. Furthermore, intra-NAc DPDPE-induced dopamine release in the NAc was partially attenuated by CTOP (Hirose et al., 2005). As we discussed above (see Figure 1.3, section on opioid receptor interactions), DPDPE is able to recruit μ-opioid receptors, through the μ-δ-opioid receptor complex or through crosstalk (Traynor and Elliot, 1993). Similarly, β-endorphin is thought to increase dopamine release in the NAc through μ-δ-opioid receptor interaction (Spanagel et al., 1990a). Enkephalins can also act through μ-δ-opioid receptor interaction (George et al., 2000). In summary, activation of μ-opioid receptors, directly or through μ-δ-opioid receptor interactions, can lead to an increase in NAc dopamine release.

We summarize this chapter as follows:

(1) Ethanol dependence is managed with naltrexone, a non-selective opioid antagonist. The involvement of the endogenous opioid systems in ethanol reinforcement is well supported by scientific evidence.

(2) Of the opioid receptors, the μ-opioid receptors play a major role in ethanol reinforcement.

(3) The mesolimbic dopamine plays a role in ethanol reinforcement.

(4) Endogenous opioid peptides activate μ-opioid receptors to increase mesolimbic dopamine release.

# Chapter 2. Rationale and Specific Aims

## ETHANOL STIMULATES THE RELEASE OF MESOLIMBIC DOPAMINE

Ethanol administration increases VTA dopamine neuron firing (Gessa et al., 1985; Brodie et al., 1990, 1999) and NAc dopamine release (Imperato and Di Chiara, 1986; Di Chiara and Imperato, 1988a; Yoshimoto et al., 1991, 1992a, b; Blanchard et al., 1993; Blomqvist et al., 1993; Heidbreder and De Witte, 1993; Campbell and McBride, 1995; Yim et al., 1998; Yim and Gonzales, 2000; Boileau et al., 2003). Also, ethanol increases NAc dopamine release dose-dependently when administered locally into NAc (Wozniak et al., 1991; Yim et al., 1998; Tuomainen et al., 2003). Due to the high concentrations required to evoke dopamine release in the NAc, the systemic effect of ethanol on NAc dopamine is probably due to an action at some other sites, including the VTA (Samson et al., 1997; Yim et al., 1998).

### Mechanisms of ethanol-stimulated mesolimbic dopamine release

Direct mechanisms

Ethanol directly activates VTA dopamine neurons (Brodie and Appel, 1998; Brodie et al., 1990, 1999). This could be due to ethanol-mediated inhibition of potassium currents (Appel et al., 2003), and voltage-gated potassium currents (m-currents) (Koyama et al., 2007). Also, ethanol targets the hyperpolarization-activated cation current ($I_h$) to increase mesolimbic dopamine neuron firing (Okamoto et al., 2006). It is important to mention that in this direct mechanism, ethanol increases VTA neuron firing, but this may not necessarily translate to enhanced dopamine release in the NAc.

Other mechanisms

Ethanol may recruit other neurochemical mechanisms in addition to the direct excitatory effects on VTA dopaminergic neurons. For example, serotonin potentiates ethanol-induced direct excitation of VTA dopaminergic neurons (Brodie et al., 1995). This could be due to ethanol stimulating serotonin 5-HT3 receptors directly (Lovinger, 1991a, b; Davies et al., 2006) and enhancing the depolarizing effects of serotonin at the 5-HT3 receptor (Lovinger and White, 1991). The 5-HT3 receptor is involved in ethanol-stimulated mesolimbic dopamine activity and release. For instance, local perfusion of a 5-HT3 antagonist blocked ethanol-induced increases in somatodendritic dopamine in the VTA (Campbell et al., 1996; Liu et al., 2006). Furthermore, systemic 5-HT3 blockade attenuates ethanol-induced dopamine release in the NAc (Carboni et al., 1989; Wozniak et al., 1990). Apart from 5-HT3 receptors, other 5-HT receptors are involved in the modulation of mesolimbic dopamine release. For example, nefazodone, a combined 5-HT2A antagonist and 5-HT reuptake inhibitor, decreases ethanol-stimulated NAc dopamine release (Olausson et al., 1998).

Apart from the serotonergic system, other neurochemical mechanisms such as cholinergic, cannabinoid and glycinergic (just to mention a few), play a role in ethanol-stimulated mesolimbic dopamine release. For instance, the systemic and intra-VTA administration of mecamylamine, a nicotinic acetylcholine receptor (nAChR) antagonist, attenuated ethanol-evoked dopamine release in the NAc (Blomqvist et al., 1993, 1997; Ericson et al., 1998, 2003, 2008; Larsson et al.,

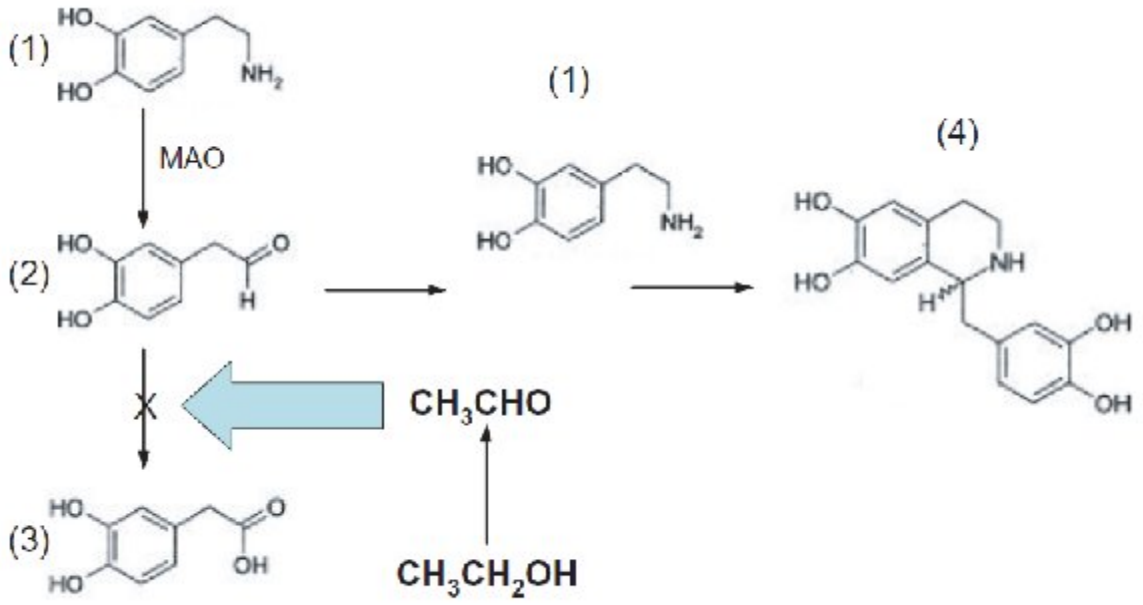
2002, 2004). Cannabinoid CB1 receptor blockade inhibited ethanol-evoked stimulation of VTA neuron firing rate (Perra et al., 2005), and other supporting studies show that ethanol-evoked mesolimbic dopamine release involves cannabinoid mechanisms (Hungund et al., 2003; Cheer et al., 2007). Ethanol can also stimulate mesolimbic dopamine through mechanisms involving the glycine receptor (Molander and Söderpalm, 2005, Molander et al. 2005). Also, there is some evidence (though not conclusive) for opioid mechanisms of ethanol-stimulated mesolimbic dopamine release (Acquas et al., 1993; Benjamin et al., 1993; Tanda and Di Chiara, 1998; Job et al., 2007). Now, we have introduced the possibility of the involvement of opioids (in addition to other non-opioid systems) in the mechanism of ethanol-stimulated mesolimbic dopamine release. In the previous chapter, we discussed the idea that endogenous opioids activate the μ-opioid receptor to modulate mesolimbic dopamine release. Ethanol may be changing the endogenous opioid system, such that this change leads an increase in mesolimbic dopamine activity. To understand this, it is important to discuss of how ethanol interacts with endogenous opioid system.

## ETHANOL ALTERS ENDOGENOUS OPIOID ACTIVITY

### Ethanol stimulates endogenous morphine-like alkaloids

Almost 40 years ago, it was suggested that ethanol may be metabolized in the body to ultimately give rise to the formation of morphine-like alkaloids called tetrahydroisoquinolines (TIQ) (Davis and Walsh, 1970a, b, Davis et al., 1970; Walsh et al., 1970; Yamanaka et al., 1970). Alcohol is metabolized by alcohol dehydrogenase to acetaldehyde. Dopamine is metabolized by monoamine oxidase to 3, 4-dihydroxyphenylacetaldehyde (refer to Figure 1.6). It is postulated

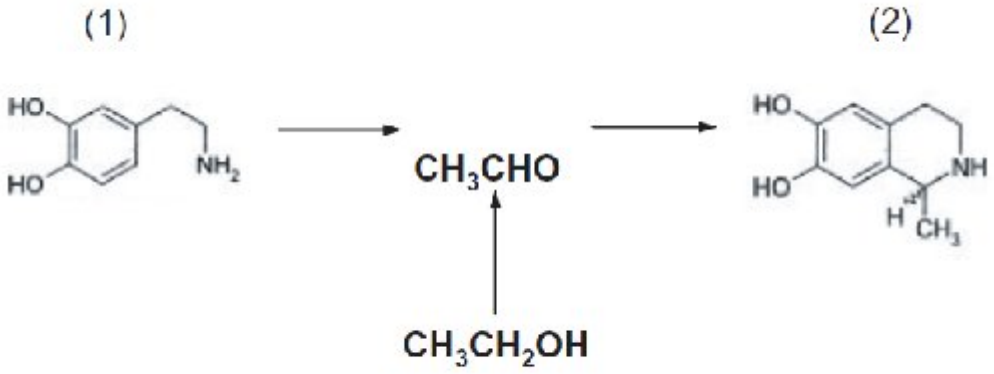
that acetaldehyde competitively inhibits the metabolism of 3, 4-dihydroxyphenylacetaldehyde to 3, 4-dihydroxyphenylacetic acid (DOPAC) by competing for acetaldehyde dehydrogenase, and the 3, 4-dihydroxyphenylacetaldehyde reacts with dopamine to give rise to tetrahydropapaveroline (THP) (Figure 2.1) (Haber et al., 1997).




(1) dopamine
(2) 3, 4-dihydroxyphenylacetaldehyde
(3) 3, 4-dihydroxyphenylacetic acid (DOPAC)
(4) tetrahydropapaveroline (THP)

**Figure 2.1**. Schematic showing the mechanism of formation of the opioid-like tetrahydropapaveroline (THP). $CH_3CH_2OH$ = ethanol, $CH_3CHO$ = acetaldehyde. Figure adapted from Haber et al. (1997).

Acute ethanol increases THP levels in the striatum and midbrain (Cashaw, 1993). However, a limitation of this study is that rats were decapitated and tissue extracted to measure THP levels 100 min after intraperitoneal ethanol administration. It is therefore not clear from this study that ethanol increases THPs. THPs affect dopamine kinetics in the NAc (Melchior et al., 1978), but it is not clear if this effect is with regards to dopamine release or dopamine re-uptake. Extensive research has been done on 1-methyl-6, 7-dihydroxy-1, 2, 3, 4-tetrahydroisoquinoline (salsolinol) (Figure. 2.2) (Jamal et al., 2003a, b). Salsolinol is formed from the condensation of dopamine and acetaldehyde (Collins and Bigdeli, 1975; Haber et al., 1997).

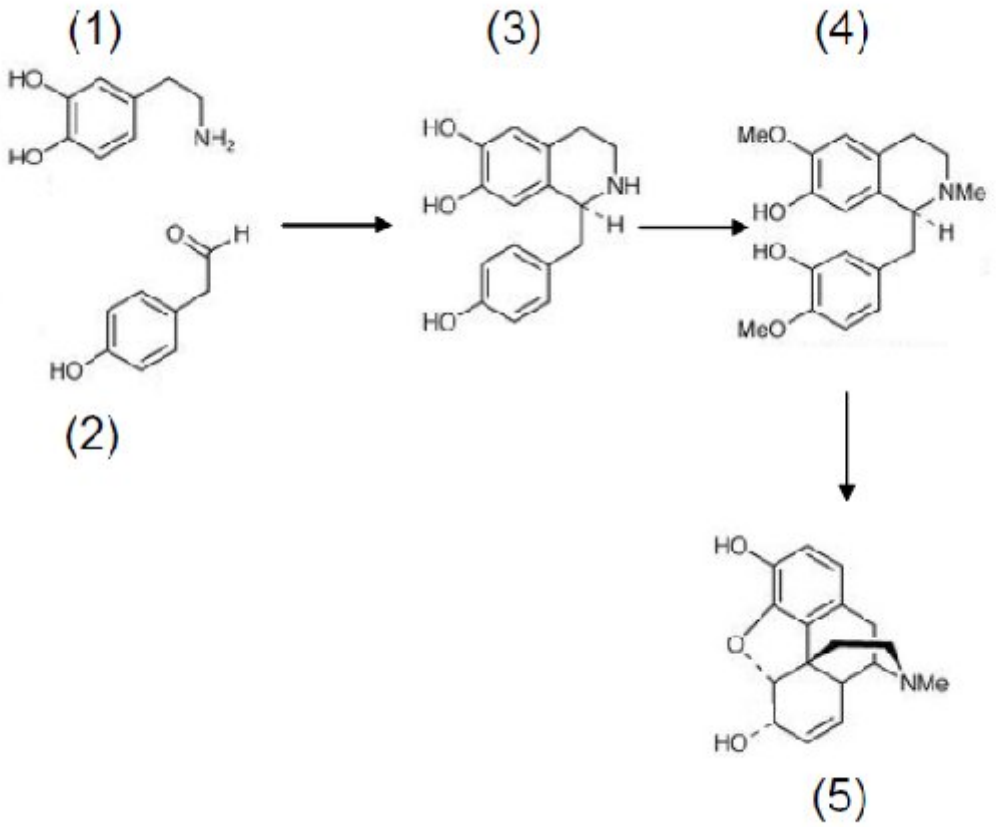



(1) dopamine
(2) salsolinol

**Figure 2.2**. Schematic showing the mechanism of formation of salsolinol. $CH_3CH_2OH$ = ethanol, $CH_3CHO$ = acetaldehyde. Figure adapted from Haber et al. (1997).

Salsolinol is found in the plasma and urine of humans after consuming ethanol (Collins et al., 1979), and is lower in the brain, at autopsy, of abstinent alcoholics than in non-abstinent alcoholics (Sjörquist et al., 1983). Salsolinol displaces met-enkephalin from its binding sites (Lucchi et al., 1982, 1985), and probably acts at μ-opioid receptors (Lucchi et al., 1985). Furthermore, salsolinol is reinforcing in the VTA (Rodd et al., 2008), suggesting that salsolinol may activate the mesolimbic dopaminergic system. However Matsubawa et al. (1987) found no correlation between blood ethanol and brain salsolinol and dopamine levels in rats. Some data shows that chronic, but not acute alcohol leads to an increase in salsolinol in dopamine rich areas of the brain such as the striatum and the limbic forebrain in rats (Hamilton et al., 1978; Sjörquist et al., 1982), though this is not very clear in other studies (Collins et al., 1990). THP and TIQ are formed in very trace amounts, and therefore their role in ethanol-stimulated mesolimbic dopamine is doubted.

There is an idea that ethanol may increase endogenous morphine (Stefano et al., 2007; Zhu et al., 2008). It has been determined that endogenous morphine synthesis is dependent on dopamine (Boettcher et al., 2005, Zhu et al., 2005; Neri et al., 2008). Dopamine combines with 4-phenylacetaldehyde to form a precursor of morphine. A summarized simplified version of the proposed mechanism of the synthesis of endogenous morphine from dopamine is shown in Figure 2.3.




(1) dopamine
(2) 4-hydroxyphenylacetaldehyde
(3) (S)-Norcoclaurine
(4) (S)-Reticuline
(5) morphine

**Figure 2.3**. Schematic showing the mechanism of formation of the morphine from dopamine. The process involves more steps than shown, but has been simplified. Me = methyl. Figure adapted from Herbert et al. (2000).

Does ethanol increase endogenous morphine release in the brain? Haber et al. (1997) determined that ethanol induces formation of morphine precursors. However, the morphine precursors (THP and S-norcoclaurine) were detected

only in the striatum, and only after long term (18 months) intake of ethanol (Haber et al., 1997). The morphine precursors were not detected in rats after 6 months alcohol intake. The idea that acute ethanol may increase endogenous morphine is not supported by any evidence. Overall, the idea that ethanol increases tetrahydroisoquinolines and endogenous morphine-like alkaloids, such that this increase contributes to the mechanism of ethanol-stimulated mesolimbic dopamine release is not supported by any evidence.

**Ethanol alters the expression of the μ-opioid receptors**

There is evidence suggesting that acute ethanol may affect the expression of μ-opioid receptors. Vukojevic et al. (2008), using the PC12 cellular model, proposed that ethanol acts by directly affecting the sorting and re-distribution of μ-opioid receptors at the plasma membrane. Also, acute ethanol changes the expression of μ-opioid receptors (Méndez et al., 2001). Mendez et al. (2001) showed that DAMGO binding was significantly decreased in the VTA and NAcS 30 min and 1 h, respectively, after ethanol administration. It is important to mention that the changes in the expression of the μ-opioid receptors could be due to changes in the release of opioid peptides. For example, acute DAMGO decreases the density of μ-opioid receptors in CHO cell line (Pak et al., 1996), therefore acute ethanol may be decreasing μ-opioid receptor density in the VTA and NAcS via release of opioid peptides active at the μ-opioid receptors. It is therefore important to discuss the interaction between ethanol and the endogenous opioid peptides.

**Ethanol stimulates the release of endogenous opioid peptides**

Ethanol affects the synthesis and regulation of β-endorphin (Gianoulakis, 1989). Acute and chronic ethanol increases β-endorphin levels in serum and in the brain (Schulz et al., 1980; Patel and Pohorecky, 1988, 1989; Anwer and Soliman, 1995). Acute ethanol increases β-endorphin content in the hypothalamus (Gianoulakis et al., 1987; Boyadjieva et al., 1999). Ethanol increases β-endorphin content in the NAc and VTA (Rasmussen et al., 1998). The ethanol-induced increase in β-endorphin in the studies mentioned above is difficult to interpret as it may reflect a product of changes in synthesis, release, transport or metabolism. However, microdialysis studies show that ethanol increases β-endorphin levels in the NAc (Olive et al., 2001; Marinelli et al., 2003, 2004) and central nucleus of the amygdala (Lam et al., 2008) in rats.

Ethanol affects the synthesis and regulation of enkephalin (Gianoulakis, 1989). Ethanol caused an increase in proenkephalin gene in the NAc (Li et al., 1998; de Gortari et al., 2000; Méndez and Morales-Mulia, 2006; Oliva et al., 2008). Also, in *utero* ethanol causes an increase in proenkephalin gene in the NAc (Druse et al., 1999). Additionally, perinatal ethanol alters met-enkephalin in rats (Lugo et al., 2006). Voluntary ethanol intake (for one month) caused a decrease in proenkephalin gene in the NAc (Cowen and Lawrence, 2001). In another study, voluntary consumption of ethanol caused an increase in met-enkephalin levels within the NAc (Nylander et al., 1994). Acute ethanol increases tissue levels of met- enkephalin in the brain (Schulz et al., 1980; Seizinger et al., 1983). However, in these tissue level studies, we cannot determine if the met-

enkephalin was released or synthesized. A better design is the microdialysis study undertaken by Marinelli et al. (2005) which shows that ethanol increases met-enkephalin levels in the NAc. Ethanol (1.6 g/kg) released met-enkephalin, and the time course of met-enkephalin release closely matches up with blood ethanol concentration, i.e. met-enkephalin peaked and declined as ethanol peaked and declined. Ethanol does not increase met-enkephalin levels in all brain structures. For instance, ethanol did not change met-enkephalin levels in the central nucleus of the amygdala (Lam et al., 2008). Heavy drinking of ethanol may decrease plasma met-enkephalin levels (Govoni et al. 1987).

We have discussed the idea that ethanol stimulates the release of endogenous opioid peptides. It is important to understand how endogenous opioid peptides are released, and to discuss how ethanol may affect this mechanism. Classical neurotransmitters (such as glutamate, GABA, acetylcholine) and neuropeptides (such as opioids) co-exist in many neurons (for review, see Hökfelt et al., 1987a, b; Merighi, 2002). The classical neurotransmitters are contained in small synaptic vesicles (SSVs), whereas the neuropeptides are contained in large dense core vesicles (LDCVs). In contrast to SSVs, LDCVs are located at some distance from the presynaptic plasma membrane (Ghijsen and Leenders, 2005). Also, the release of SSVs takes place at the active synaptic zones, whereas the release of LDCV content takes place at remote sites different from the synapse (Karhunen et al., 2001; Ghijsen and Leenders, 2005). In general, low frequencies of stimulation lead to the release of the classical neurotransmitters. The neuropeptide is released only after higher frequencies (or more prolonged bursts of low frequencies) of stimulation than is necessary for the exocytotic release of

the neurotransmitters (Whim and Lloyd, 1989; Leenders et al., 1999; Ghijsen and Leenders, 2005). After depolarization of a neuron, there is a fast release of classical neurotransmitter and a slower exocytosis of neuropeptide (Leenders et al., 2002; Ghijsen and Leenders, 2005). A summary of the proposed delayed effect of opioid peptides is shown in Figure 2.4.

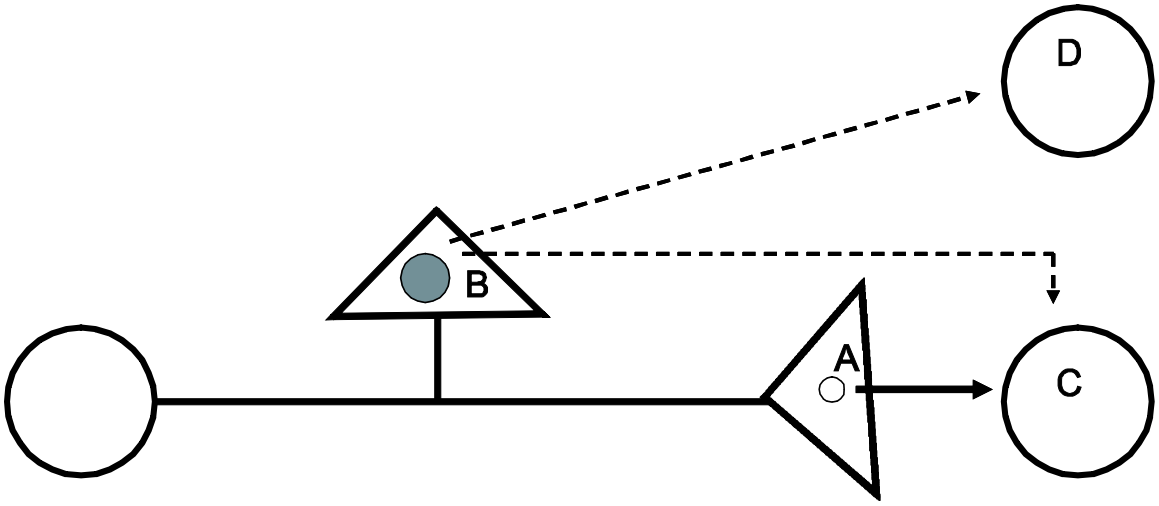



**Figure 2.4**. Schematic showing the release of peptides compared to classical neurotransmitters. **A** = small synaptic vesicle containing classical neurotransmitter, **B** = large dense core vesicle containing neuropeptide, **C** = postsynaptic target, **D** = distant target. The classical neurotransmitter undergoes fast exocytotic release and travels a relatively short distance to the postsynaptic target. The peptide undergoes slower exocytotic release at sites remote from the synaptic cleft, and therefore has to diffuse through some distance before it reaches the postsynaptic target or a distant target. Overall, there is a delay in the effect of the neuropeptide.

Apart from depolarization of opioidergic neurons, opioids may also be released by the action of other neurotransmitters. Adenosine increases β-endorphin release from the hypothalamus (Boyadjieva and Sarkar, 1999). Also, dopamine can increase β-endorphin levels in the NAc (Roth-Deri et al., 2003). Acute administration of nicotine increases met-enkephalin release in the NAc (Houdi et

al., 1991). Serotonin (5HT) increases the extracellular levels of β-endorphin in the arcuate nucleus of the hypothalamus and in the NAc (Zangen et al., 1999).

An important question is therefore 'how does ethanol release the endogenous opioid peptides?' The mechanism of ethanol-stimulated increase of β-endorphin from the hypothalamus involves many voltage-gated calcium channels (P/Q, N, L, and T) (De et al., 1999). Boyadjieva and Sarkar (1999) determined that ethanol increases β-endorphin by increasing adenosine, which via adenosine receptors, increases β-endorphin. Based on discussions in the previous paragraph, it is possible that ethanol also increases endogenous opioids through activation of dopaminergic, cholinergic, and serotonergic systems. More research needs to be done to establish the mechanism of endogenous opioid peptide release via ethanol.

## OPIOID MECHANISMS OF ETHANOL-STIMULATED MESOLIMBIC DOPAMINE RELEASE

### Is there a link between ethanol-stimulated endogenous opioid release and ethanol-stimulated mesolimbic dopamine release?

There is a general idea that ethanol stimulates endogenous opioids as part of the mechanism of ethanol-evoked mesolimbic dopamine release. For instance, it has been suggested that ethanol stimulates β-endorphin release, and this released β-endorphin acts on μ- (and δ-opioid) receptors in the VTA and/ or NAc to give rise to an increase in mesolimbic dopamine (Herz, 1997). However, closer examination of the microdialysis data (Olive et al., 2001; Marinelli et al., 2003, 2004) shows that ethanol-stimulated β-endorphin release in the NAc does not

match up with the typical time course for ethanol-stimulated dopamine release. Microdialysis detects an increase in β-endorphin levels only at a time *after*, and not before, dopamine increase in the NAc. There are numerous data showing a delay ($\geq$ 60 min) in β-endorphin release in the NAc (measured via microdialysis) after acute ethanol administration (Olive et al., 2001; Marinelli et al., 2003, 2004; Lam et al., 2008). From this, we conclude that opioid mediated increase in dopamine is probably not due to β-endorphin release and activity in the NAc. In fact, evidence seems to suggest that β-endorphin in the NAc is under dopaminergic control (Roth-Deri et al., 2003). With regards to ethanol-stimulated mesolimbic dopamine release, a probable target for β-endorphin action is the VTA. Indeed, preliminary studies show that acute ethanol administration increases β-endorphin release in the VTA (Jarjour and Gianoulakis, 2006, abstract).

From microdialysis data, ethanol stimulated met-enkephalin increase within 30 min after ethanol administration in the NAcS (Marinelli et al., 2005) and VTA (Jarjour and Gianoulakis, 2006, abstract). This increase is detectable at such a time that it may correspond to ethanol-stimulated dopamine increases. However in these studies, sampling interval was 30 min, making it difficult therefore to estimate the nature of the time course of this release. It has been suggested that this ethanol-mediated increase in met-enkephalin in the NAc and VTA may activate μ- and δ-opioid receptors to give rise to an increase in mesolimbic dopamine.

It is unclear why β-endorphin and enkephalin release is delayed. One possible explanation is that when opioids are released, they have to diffuse through some distance to act at their targets (Figure 2.4). In summary, there may be a link between ethanol-stimulated opioid and dopamine release, but this is not clear, and more research needs to be done to clarify this.

**A closer look at studies investigating the opioid mechanisms of ethanol-stimulated mesolimbic dopamine release**

A look into the literature shows some studies have been done to find an opioid mechanism of ethanol-mediated mesolimbic dopamine release. For instance, in behavioral studies, naltrexone, a non-selective opioid antagonist, suppresses dopamine increase due to ethanol self-administration (Gonzales and Weiss, 1998). However, naltrexone also suppressed ethanol intake in 6-OHDA-lesioned rats (Koistinen et al., 2001), suggesting that dopamine is not required for naltrexone suppression of ethanol reinforcement. Similarly, naloxone inhibited ethanol intake in rats with 6-OHDA lesions of the mesolimbic pathway (Shoemaker et al., 2002). In *in situ* hybridization studies, chronic ethanol consumption increased tyrosine hydroxylase mRNA levels in the VTA, and naltrexone (1 mg/kg) treatment suppresses the ethanol-induced increase in the VTA tyrosine hydroxylase mRNA levels (Lee et al., 2005).

Benjamin et al. (1993) reported that naltrexone reversed ethanol-induced dopamine release in the NAc in awake, freely moving Long Evans rats. However,

in this paper (Benjamin et al, 1993), very high ethanol concentration (5%v/v, >800 mM) was locally administered (via reverse dialysis) into the NAc throughout the experiment (200 min), and the changes in mesolimbic dopamine were measured, with naltrexone (systemic) cumulatively (0, 0.25, 0.5, and 1 mg/kg i.p.) administered. In this study, the ethanol was continuously infused into the NAc, then two 20 min samples were taken, after which saline different doses of naltrexone were injected 40 min apart (to allow the collection of two 20 min samples after each injection). Details regarding the recovery of the probes, and the approximate amount of ethanol reaching the NAc were not given, and thus it is impossible to estimate how much ethanol may have reached the NAc. In addition to this, when ethanol is administered via reverse dialysis into the NAc, its concentration in brain tissue may vary dramatically with distance from the probe (Wozniak et al., 1991). This makes conclusions regarding an estimate of the amount of ethanol reaching the NAc difficult. Also, when ethanol is administered into a specific brain structure like the NAc, as opposed to systemic administration, the biological effects have to be carefully interpreted. For example, some other studies involving focal application of ethanol into the NAc show that the NAc is relatively insensitive to the dopamine stimulating effects of ethanol, except when very high concentrations of ethanol are used (Wozniak et al., 1991; Yim et al., 1998; Tuomainen et al., 2003).

Following systemic administration, the concentration of ethanol reaching the brain peaks and then decreases as the ethanol is metabolized and undergoes clearance. In this study, continuous intra-NAc administration of ethanol was given, introducing a question as to whether the measured dopamine response can ever be attained physiologically. For example, in other studies utilizing reverse dialysis of ethanol into the NAc, the ethanol-evoked dopamine response is not continuous throughout the administration of ethanol (Tuomainen et al., 2003; Lof et al., 2007). In the study by Lof et al. (2007), they showed that the NAc dopamine increases due to focally administered ethanol last only about 40 min, regardless of continuous intra-NAc ethanol infusion. Another example can be seen from the work by Tuomainen et al. (2003) in which they found that a reverse dialysis of 800 mM of ethanol continuously for 1 h into the NAc of rats increased dopamine, but the dopamine peaked at 15 min and was back to baseline at around 45 min, even while the ethanol was still continuously administered. Going back to the paper by Benjamin et al. (1993), it is likely, therefore, that at time > 40 min after the start of intra-NAc ethanol infusion (and before the injection of the naltrexone doses) there may be a time-dependent decrease in dopamine release (see Tuomainen et al., 2003; Lof et al., 2007). This implies that, absent any appropriate controls, the observed changes in dopamine may be due to a time-related decrease in ethanol-induced dopamine release and not due to naltrexone administration. An appropriate control would have been to repeat the same experiment, substituting saline for all corresponding cumulative naltrexone injection doses, in order to identify if there is a time-dependent change in ethanol-induced dopamine throughout the duration of the experiment (200 min) independent of naltrexone administration.

Also in this experiment, rats were injected i.p. 4 times with saline or various naltrexone doses. This procedure is stressful, and it is thought that stress can affect endogenous opioid levels (Patel and Pohorecky, 1988; Marinelli et al., 2004). Furthermore, there may be an involvement of endogenous opioid mechanisms in the interaction between stress and ethanol (Trudeau et al., 1991). We, therefore, cannot exclude stress as a confounding variable in the results obtained in the experiment. An adequate control to address this has been mentioned above, or alternatively a less stressful route of administration can be used. A typical dopamine response includes an initial increase, and then a decrease back to baseline. In this experiment, the initial increase in dopamine was supposedly sustained throughout the experiment, and decreased by the cumulative doses of naltrexone. With all the limitations mentioned above, we cannot conclude with confidence, that naltrexone attenuates ethanol-evoked dopamine release in the NAc, and a better experimental design needs to be done.

Inoue (2000) examined the effect of naltrexone (systemic) on the ethanol (systemic) evoked increases in the firing rates of VTA dopaminergic neurons in anesthetized male Sprague Dawley rats. The conclusion of this research work (Inoue, 2000) is that naltrexone attenuates ethanol-evoked increases in VTA dopaminergic neuron firing, but only at very high naltrexone doses (30 mg/kg). In any case, 3.0 mg/kg of naltrexone, which is a pharmacologically relevant dose, had no effect on ethanol induced VTA dopamine neuron firing activity. This data can be interpreted to mean that, notwithstanding the administration of 3.0 mg/kg

naltrexone, ethanol still increased the firing of VTA dopamine neurons. One limitation is that VTA dopamine neuron firing does not necessarily correspond to a release of dopamine in the NAc. Also, even though this research data concluded that naltrexone resulted in depression of VTA dopaminergic neuronal firing rates activated by acute ethanol administration in the NAc, the doses of naltrexone (30 mg/kg) which achieved this were extremely high and may have achieved this through non-opioid mechanisms.

In separate experiments in this study (Inoue, 2000), the accumulation of L-DOPA (which is considered as an indicator of dopamine metabolism or release) in the NAc after ethanol administration was measured. Ethanol 2.5 g/kg (i.p.) caused approximately 40% increase in L-DOPA in the NAc. Naltrexone 3.0 and 10.0 mg/kg were ineffective in suppressing the accumulation of L-DOPA in the NAc due to 2.5 g/kg ethanol. A major limitation of this work is that rats were decapitated, and the NAc was extracted for L-DOPA analysis, 90 min after ethanol administration. However, in other microdialysis studies, ethanol (2.5 g/kg, i.p.) increases dopamine in the NAc shortly after administration, and the dopamine levels are back to baseline at 60 min after ethanol administration (Blomqvist et al., 1993, 1997; Olausson et al., 1998). L-DOPA accumulation in the NAc was therefore analyzed *after* dopamine levels in the NAc would have come back to baseline. We therefore cannot correlate the L-DOPA levels with ethanol-mediated dopamine release. The Inoue (2000) study showed that naltrexone (30 mg/kg), but not naltrexone (3 and 10 mg/kg), was effective in suppressing ethanol-mediated increase in VTA dopamine neuron firing and ethanol-mediated accumulation of L-DOPA in the NAc. With the limitations of this

study, it is not clear if naltrexone suppresses acute ethanol-mediated increase in mesolimbic dopamine release

It is also not clear if the μ-opioid receptors play a role in the mechanism of ethanol-stimulated dopamine release. Ethanol-stimulated mesolimbic dopamine release is attenuated in some, but not all, models of the μ-opioid receptor knockout mice (Job et al., 2007). A major limitation with the knockout studies is that lifelong deletion of a gene can lead to compensatory mechanisms and therefore data obtained from knockout models should be interpreted very carefully, preferably with some pharmacological antagonism studies supporting the findings. Naloxonazine (15 mg/kg i.p.), an irreversible selective μ1-opioid antagonist, suppresses ethanol-induced mesolimbic dopamine release (Tanda and Di Chiara, 1998) in freely-moving animals, suggesting that the μ1-opioid receptor is involved in this mechanism. However, a limitation of naloxonazine is that it is thought to block δ-opioid receptors (Dray and Nunan, 1984), and since δ-opioid receptors may be involved in ethanol-induced dopamine release in the NAc (Acquas et al., 1993), it may be argued that the effect may be due to μ1- or δ-opioid receptors or both. However, naloxonazine has a 200 fold higher selectivity of μ1- over δ-opioid receptors (Raynor et al., 1994). Honkanen et al. (1996) showed that doses of naltrindole which block δ-opioid function had no effect on ethanol intake whereas naloxonazine (15 mg/kg i.p.) did, suggesting that the effects of naloxonazine at this dose are predominantly due to μ1-opioid receptors. We believe that naloxonazine is acting predominantly at the μ1- opioid receptor (see discussion on naloxonazine in section on the pharmacology of the μ-opioid antagonists). Therefore, the idea that naloxonazine is acting through the

δ-opioid is not very strong, making a case for a role of μ-opioid receptors in ethanol-evoked dopamine release in the NAc (Tanda and Di Chiara, 1998). However, naloxonazine is not effective in suppressing ethanol-stimulated mesolimbic dopamine in all studies (see Job et al., 2007). In summary, it is not clear that μ-opioid inhibition leads to an attenuation of ethanol-stimulated mesolimbic dopamine release (Tanda and Di Chiara, 1998; Job et al., 2007).

**Proposed mechanism of μ-opioid-mediated ethanol-stimulated mesolimbic dopamine release**

Ethanol increases endogenous opioids (Olive et al., 2001; Marinelli et al., 2003, 2004, 2005, 2006). Endogenous opioids, such as enkephalin (Daugé et al., 1992; Cador et al., 1989; Kalivas and Richardson-Carlson, 1986), β-endorphin (Iyenger et al., 1989; Spanagel et al., 1990a, 1991a, b), endomorphin (Huang et al., 2004; Okutsu et al., 2006; Terashvili et al., 2008) all increase dopamine release in the NAc. It is hypothesized that ethanol increases dopamine, in part, by stimulating endogenous opioid peptides which in turn act at μ-opioid receptors to increase dopamine in the NAc (Figure. 2.5). Based on the scheme in Figure.2.5,

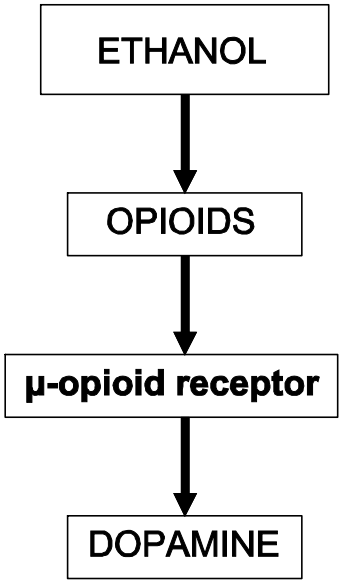



**Figure 2.5**. Schematic showing proposed μ-opioid mechanism of ethanol-stimulated mesolimbic dopamine release.

therefore, blockade of the μ-opioid receptors should lead to a suppression of ethanol-stimulated mesolimbic dopamine release.

**SUMMARY AND SPECIFIC AIMS**

We discussed data showing that ethanol increases dopamine release in the NAc. Also, ethanol stimulates the release of endogenous opioid peptides. It is proposed that ethanol increases mesolimbic dopamine, in part, by stimulating the release of endogenous opioids to activate the μ-opioid receptor, leading to a disinhibition of VTA dopaminergic neurons to result in an increase in dopamine in the NAc. We ended the review, by discussing experiments that had been done previously to determine the role of the μ-opioid receptor in the mechanism of ethanol-induced mesolimbic dopamine release. Previous experiments concluded that the μ-opioid receptors play a role in ethanol-evoked mesolimbic dopamine release. After a careful look into the literature regarding previous experimental designs and limitations, we are convinced that the conclusions need to be interpreted with care, and we are convinced that more comprehensive research needs to be done to effectively characterize the role of the μ-opioid receptor in the mechanism of ethanol-evoked dopamine release in the NAcS, and we propose such an experiment.

For the purposes of this project, we will study the involvement of the μ-opioid receptor in ethanol-induced dopamine release in the NAcS in male rats. In our experiments, Long Evans rats will be used, and dialysate dopamine and ethanol will be obtained using *in vivo* microdialysis, and analyzed using HPLC and GC, respectively. Our hypothesis is that the μ-opioid receptor plays a role in ethanol-

stimulated dopamine release in rats; therefore blockade of the μ-opioid receptor should decrease this effect. This hypothesis will be tested by the following specific aims:

(1) To find out if naltrexone affects basal dopamine levels in the NAcS. This will be done by administering cumulative intravenous (i.v.) doses of saline and different doses of naltrexone to determine if there is a change in dopamine in the NAcS.

(2) To experimentally find an effective dose of naltrexone to suppress morphine-evoked dopamine release in the NAcS. We will look for a dose-dependent effect of systemic naltrexone on morphine-stimulated dopamine release in the NAcS. This will be done by i.v. pretreatment of rats with vehicle or different doses of naltrexone and subsequently measuring morphine-stimulated dopamine release in the NAcS.

(3) To measure the effect of systemic naltrexone on ethanol-stimulated dopamine release in the NAcS. This will be done by i.v. administration of vehicle, or a dose of naltrexone that is effective in abolishing morphine-evoked NAcS dopamine release, and determining the effect of these effective doses on ethanol-stimulated dopamine release.

(4) To measure the effect of a specific μ-opioid antagonist on morphine-stimulated dopamine release. This will be done by subcutaneous (s.c.) pretreatment of rats with vehicle or β-funaltrexamine, and subsequently

measuring dopamine release in the NAcS due to i.v. morphine administered 20-24 hours later.

(5) To measure the effect of a specific μ-opioid antagonist on ethanol-stimulated dopamine release in the NAcS of Long Evans rats. This will be done by s.c. pretreatment of rats with vehicle or the dose of β-funaltrexamine that attenuated morphine-evoked dopamine release in the NAcS (in previous experiment), and subsequently measuring dopamine release in the NAcS due to i.v. ethanol administered 20-25 h later.

# Chapter 3. The role of the μ-opioid receptors in the mechanism of ethanol-stimulated dopamine release in the nucleus accumbens shell in ethanol-naïve rats


**Abstract**

**Background**: It has been proposed that the μ-opioid receptor plays a role in ethanol-reinforcement through modulation of the mechanism of ethanol-stimulated mesolimbic dopamine release. Our hypothesis is that blockade of the μ-opioid receptors attenuate ethanol-mediated increases in dopamine release in the nucleus accumbens shell.

**Methods**: Ethanol-naïve male Long Evans rats (n = 95) were prepared for *in vivo* microdialysis and intravenous drug infusion. Experiments were done to determine the effect of naltrexone and β-funaltrexamine on morphine- and ethanol-stimulated dopamine release in the nucleus accumbens shell.

**Results**: Naltrexone and β-funaltrexamine, via blockade of the μ-opioid receptors, suppressed the prolongation, but not the initiation of the dopamine release by ethanol.

**Conclusions**: The μ-opioid receptors are involved in a delayed component of ethanol-stimulated dopamine release in the nucleus accumbens shell in ethanol-naïve rats.



**Key words**: ethanol, morphine, naltrexone, β-funaltrexamine, mesolimbic dopamine

## Introduction

The mesolimbic dopaminergic system, which includes the pathway from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), is thought to play a role in ethanol reinforcement (for review, see Gonzales et al., 2004). Endogenous opioidergic systems are also thought to play a role in ethanol reinforcement (for review, see Herz, 1997). Naltrexone, a non-selective opioid antagonist, is FDA approved for clinical use in the management of alcoholism (Volpicelli et al., 1992, 1995; O'Brien et al., 1996). Also, β-funaltrexamine, a selective μ-opioid antagonist, is effective in suppressing ethanol intake in rats (Krishnan-Sarin et al., 1998; Stromberg et al., 1998a), and there is a strong suggestion that the μ-opioid receptors play a major role in ethanol intake and reinforcement (Myers & Robinson, 1999; Roberts et al., 2000; Hall et al., 2001; Hyytia and Kiianmaa, 2001; Becker et al., 2002; Lasek et al., 2007). It has been proposed that blockade of the μ-opioid receptors leads to the suppression of ethanol reinforcement partly by attenuating ethanol-stimulated mesolimbic dopamine activity (Herz, 1997).

Naltrexone, in an operant self-administration study, attenuated the NAc dopamine increase that occurs as a result of ethanol self-administration (Gonzales and Weiss, 1998) in ethanol-experienced rats. However, in another behavioral study in ethanol-experienced rats, naltrexone suppression of ethanol intake was retained in ethanol-experienced rats with lesions of the mesolimbic dopamine pathway (Koistinen et al., 2001) suggesting that the mechanism involved in naltrexone suppression of ethanol reinforcement may not even

require mesolimbic dopamine. It is also not very clear if naltrexone attenuates acute ethanol-mediated increases in mesolimbic dopamine activity in ethanol-naïve rats. For instance, even though Benjamin et al. (1993) reported that, in ethanol naïve rats, naltrexone attenuated ethanol-induced dopamine release in the NAc, Inoue (2000) reported that naltrexone, at pharmacologically relevant doses, did not decrease acute ethanol-induced increase in the firing rates of VTA dopaminergic neurons in anesthetized ethanol-naïve rats. More research needs to be done to clarify the effect of naltrexone on ethanol-stimulated mesolimbic dopamine release. Naltrexone is non-selective, blocking μ-, δ- and κ-opioid receptors. The opioid receptors are involved in the modulation of mesolimbic dopamine (Herz, 1997). Therefore, it is important to characterize the respective contribution of the individual receptor types to the effect of naltrexone on ethanol-stimulated dopamine release.

A literature search shows that some experiments to characterize the contribution of the μ-opioid receptor to the mechanism of ethanol-stimulated mesolimbic dopamine release have been done. For instance, Tanda and Di Chiara (1998) reported that naloxonazine, an irreversible selective μ1-opioid receptor subtype antagonist, suppressed ethanol-induced mesolimbic dopamine release. However, naloxonazine did not block ethanol-stimulated mesolimbic dopamine release in all animal models (Job et al., 2007). Furthermore, naloxonazine is thought to also block δ-opioid receptors (Dray and Nunan, 1984), and there is evidence that δ-opioid receptors play a role in the mechanism of ethanol-stimulated mesolimbic dopamine release (Acquas et al., 1993). The role of the μ-

opioid receptor in the mechanism of ethanol-evoked mesolimbic dopamine release is not clear, and more work needs to be done to clarify this.

*In vivo* microdialysis shows that intravenous (i.v.) ethanol increases dopamine in the nucleus accumbens shell (NAcS) in awake, freely moving naïve Long Evans rats (Howard et al., 2008). In the present study, our goal was to test the hypothesis that in naïve rats, blockade of the μ-opioid receptors inhibits ethanol-mediated increases in dopamine release in the NAcS. To test this hypothesis, we prepared male Long Evans rats for i.v. drug administration and intracerebral microdialysis from the NAcS and examined the effect of naltrexone (i.v.) and β-funaltrexamine (s.c.) pretreatments on morphine (positive control experiments) and ethanol-evoked increases in dopamine release.

## Materials and Methods

### *Animals*

Male Long-Evans rats (n = 95) (Charles River Laboratories, Wilmington, MA), weighing 280-407 g on dialysis day, were used for these experiments. Sixty-nine and twenty-six rats were used for the naltrexone and β-funaltrexamine experiments, respectively. The rats were housed individually in a temperature (25˚C) and light (12 h light/12 h dark) controlled room, and had access to food and water *ad libitum*. The rats were handled for at least four days prior to surgery. All procedures were carried out in compliance with the guidelines set forth by the *National Institutes of Health Guide for the Care and Use of*

*Laboratory Animals* and the Institutional Animal Care and Use Committee of the University of Texas at Austin.

*Surgery*

A jugular catheter was inserted, and a guide cannula was placed over the NAc in each rat using the procedure of Howard et al. (2008). The catheter was placed in the jugular vein, passed subcutaneously to exit an incision on the head. Intravenous catheters were constructed from silastic tubing (0.30 mm ID, 0.64 mm OD, 0.15 mm wall thickness, Fisher Scientific, Hampton, NH), a cannula (22 gauge, Plastics One, Roanoke, VA), and silicon adhesive (DAP Inc., Baltimore, MD). The rats were under isoflurane anesthesia (2.0%) during stereotaxic and jugular catheterization surgery. The guide cannula used for microdialysis (21 gauge, Plastics One, Roanoke, VA) was implanted above the shell (coordinates in mm relative to Bregma: AP +2.2, ML +0.9, DV -3.8) of the NAc while the animal was in a stereotaxic frame. The DV coordinate represents the bottom of the guide cannula, and the probe extends an additional 4.0 mm below the cannula when seated into the guide. An obturator was placed in the guide cannula to prevent blockage. Rats were allowed to recover from surgery for 3-6 days before experiments.

*Microdialysis*

The evening before the dialysis experiment, a laboratory constructed probe (1.5 mm active membrane length, 270 μm OD, 18,000 molecular weight cut-off) was implanted through the guide cannula and perfused (CMA 100 microinjection pump, Acton, MA) with artificial cerebrospinal fluid (ACSF: 149 mM NaCl, 2.8 mM

KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.25 mM ascorbic acid, 5.4 mM D-glucose). The rats were placed in individual chambers with free access to water and food, and the flow rate was lowered to 0.2 µL/min, and allowed to run overnight. The next morning, the flow rate was increased to 2.0 µL/min., and allowed to run at this flow rate for two hours before the dialysis experiment began. Samples were collected at 5-min intervals. In one experiment, naltrexone was cumulatively administered i.v. to determine the effect of naltrexone on basal dopamine levels. In another experimental design, naltrexone was administered i.v. 20 min before i.v. drug (morphine, ethanol and saline) treatment. In these experiments, morphine (1 mg/kg) was delivered as a bolus dose, ethanol (1g/kg) and equivalent volume saline treatments were delivered at the rate of 4 mL/min. In yet another experimental design, β-funaltrexamine was administered s.c. 20-25 h before rats were given i.v. drug treatments (morphine, ethanol and saline). In these experiments, all animals received saline, followed by either morphine or ethanol. Upon completion of the experiments, the perfusate was switched to calcium-free ACSF. A 5-min sample was taken after 1 h to verify that dopamine recovered in the experimental samples was due to calcium-dependent exocytotic release. For all groups, every dialysis sample was analyzed for dopamine, and ethanol was determined in the post-infusion samples in the ethanol experiments.

*Histology*

After the dialysis experiment (1-3 days), the rats were overdosed with sodium pentobarbital (150 mg/kg, i.v.). The rats were perfused intracardially with saline followed by 10% formalin, and the brains were extracted and placed in 10% formalin overnight. The brains were sectioned (100 µm thick) with a Vibratome

(Leica, Nussloch, Germany) and then stained with cresyl violet to confirm probe placement in the NAcS. The probe tracks were mapped using the atlas of Paxinos and Watson (1986), and Paxinos et al. (1999).

*High Pressure Liquid Chromatography (HPLC) and Gas Chromatography (GC)*
Dialysate dopamine was analyzed using HPLC with electrochemical detection. The system used a Polaris 3µ C18 column (50 x 2 mm, Varian, Lake Forest, CA). The mobile phase (pH = 5.6) consisted of 0.50 g octanesulfonic acid, 0.05 g decanesulfonic acid, 0.13 g ethylenediaminetetraacetic acid, 11.1 g $NaH_2PO_4$, and 150 ml methanol, all in 1 L of deionized water. Dialysate samples were mixed with ascorbate oxidase at 4˚C prior to injection. Dopamine was detected with an electrochemical detector (Model VT03, Antec Leyden, Netherlands) at a potential of + 345 mV (relative to an Ag/AgCl reference). A second system was used for some samples in which the reference was an *in situ* Ag/AgCl (ISAAC). KCl was added to the mobile phase in appropriate concentrations in this case (4.47 g/L). The limit of detection was ~0.2 nM. The peaks were recorded using EZChrom software, and the concentration of dopamine in each sample was determined using external standards.

Ethanol was analyzed in 2 µl aliquots that were transferred from the dialysate sample into 2 ml gas chromatography vials immediately after sample collection. Dialysate ethanol concentrations were determined following the method of Howard et al. (2008). A Varian CP 3800 gas chromatograph with flame ionization detection and a Varian 8200 headspace autosampler was used to analyze the concentrations of ethanol in the samples. The stationary phase was an HP

Innowax capillary column (30 m x 0.53 mm x 1.0 μm film thickness) and helium was the mobile phase. Resulting ethanol peaks were recorded using Varian Star Chromatography Workstation software, and calibration was achieved using external standards.

*Drugs and drug treatments*

Morphine sulphate salt pentahydrate (NIDA Drug Supply Program) was dissolved in saline (1 mg/mL). Naltrexone hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in saline. β-funaltrexamine hydrochloride (Tocris Bioscience, Ellisville, MO) was dissolved in de-ionized water and appropriate NaCl was added to the resultant solution to make it 0.9%w/v (normal saline). A 10% w/v solution of ethanol in saline was made from ethanol (95%) (Aaper Alcohol and Chemical Co., Shelbyville, KY). All drugs were administered via the i.v. route except β-funaltrexamine which was administered via the s.c. route.

*Data Analysis*

The dopamine data was either used directly (naltrexone cumulative experiment) or transformed to percent of basal values (all other experiments). The Area Under the Curve (AUC), which is the difference between post-infusion dopamine levels and baseline, was calculated for dopamine data for some experiments. The analysis of the dopamine time course was done using repeated measures ANOVA using SPSS (SPSS Inc, Chicago, IL). The analysis of the AUC was done using One-way ANOVA using SPSS. The criterion for type I error was set to $P < 0.05$. We performed post hoc contrasts (Bonferroni corrected), comparing individual time points between treatment groups, and individual time points with

basals within groups when significance was detected for time, or drug treatment x time interaction. Values are reported as mean ± SEM.

For all experiments, the within subject variable was time. For the experiment involving naltrexone and morphine, the between subject variable was naltrexone dose (4 levels). For the naltrexone and ethanol experiment, we have one between subject factor (naltrexone dose, with 3 levels). For experiments involving β-funaltrexamine, the between subject variable was β-funaltrexamine (2 levels). For all experiments, there were 20 missing data points (out of a total of 1510) due to problems with chromatography or sample collection. The missing points were estimated by taking the average of the values on either side of the missing point and the degrees of freedom in the final statistical analyses were corrected for the estimated data.

**Results**

*Histology and calcium dependency of dialysate dopamine*

The microdialysis probe placements in the NAcS are shown in Figure 3.1. Rats that had probes (a) completely in the NAcS (89% of rats) and (b) predominantly in the NAcS with not more than 35% of the probe in the border between the NAc shell and the core (11% of rats) were included in the analysis.

The $Ca^{2+}$ dependency of dialysate dopamine was ascertained for all rats used in this study. The criterion for acceptable $Ca^{2+}$ dependency was the attainment of at least 60% reduction in extracellular dopamine during $Ca^{2+}$ free ACSF perfusion

compared with regular ACSF perfusion. For all experiments, the mean reduction for extracellular dopamine was 85 ± 2%.

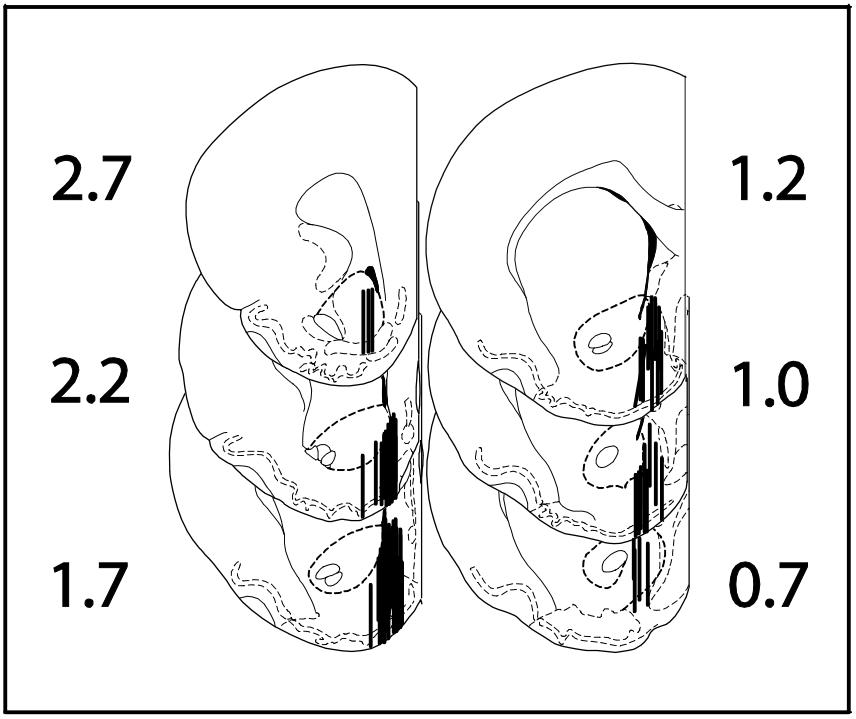



**Figure 3.1**. Histological analysis to confirm probe placements in the nucleus accumbens shell. Coordinates are from Bregma.

*Naltrexone does not alter basal levels of dopamine.*

We did three naltrexone experiments. One experiment involved cumulative i.v. administration of saline and different doses of naltrexone (0.125, 0.25, 0.5 mg/kg). Another experiment involved i.v. infusion of saline or different doses of naltrexone (0.01, 0.1, and 0.3 mg/kg) 20 min before infusion of a single dose of morphine. Yet another experiment involved i.v. infusion of saline or different doses of naltrexone (0.3 and 1.0 mg/kg) 20 min before infusion of ethanol or

saline. For all experiments, saline infusions (controls) did not cause any change in basal dopamine levels. In the naltrexone cumulative dosing experiment, naltrexone did not cause any change in dopamine basal levels ($F_{19, 94} = 1.06$, $P > 0.05$, time) (Figure 3.2).

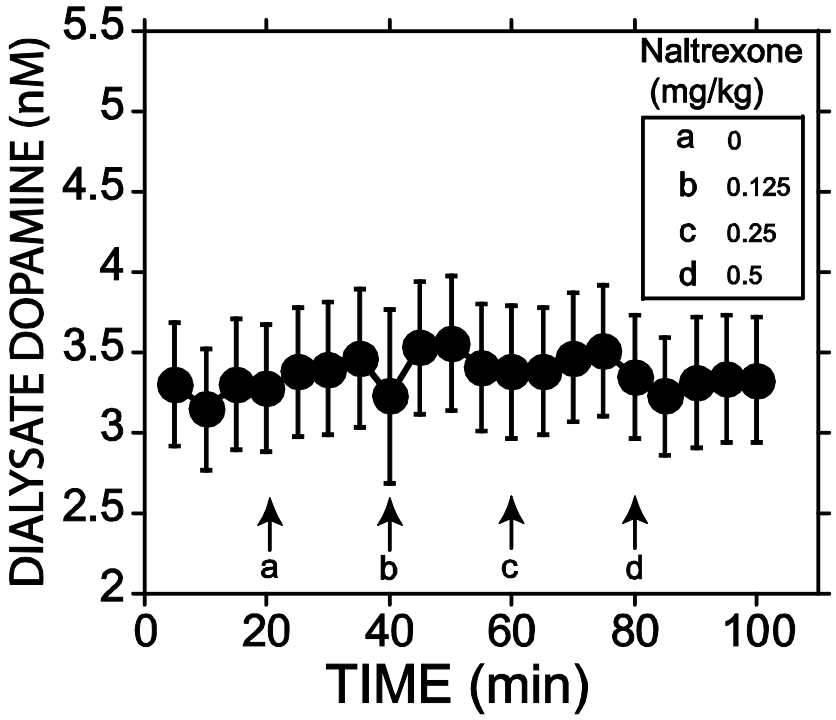



**Figure 3.2**. Naltrexone does not change basal levels of dopamine. This shows the dopamine response in the shell of the nucleus accumbens of male Long Evans rats after i.v. pretreatment with cumulative doses of naltrexone (n = 6). Each rat received saline and naltrexone doses (0.125, 0.25, and 0.5 mg/kg), with infusions administered 20 min apart. The arrows indicate the point of infusion.

In the naltrexone and morphine experiment, naltrexone (0.01, 0.1 and 0.3 mg/kg) did not alter basal dopamine levels. ANOVA showed no significant difference in dopamine levels after infusion of saline (n = 6) or naltrexone doses (mg/kg): 0.01 (n = 5), 0.1 (n = 6), and 0.3 (n = 7) ($F_{15, 99} = 0.69$, $P > 0.05$, for the naltrexone dose x time interaction). Before naltrexone pretreatment, basal dopamine levels

(nM) were 1.0 ± 0.3, 1.9 ± 0.4, 1.8 ± 0.5, and 1.2 ± 0.4 for saline and naltrexone doses 0.01, 0.1, and 0.3 mg/kg respectively. In the naltrexone and ethanol experiment, naltrexone (0.3 and 1.0 mg/kg) did not alter basal dopamine levels. ANOVA showed no significant change in dopamine levels after saline (n = 12), naltrexone 0.3 mg/kg (n = 14), or 1.0 mg/kg (n = 13) infusions ($F_{14, 248} = 1.25$, $P > 0.05$, for the naltrexone dose x time interaction). For these experiments basal dopamine levels (nM) (before naltrexone infusion) were 1.0 ± 0.2, 1.2 ± 0.2, and 1.2 ± 0.2 respectively.

*Naltrexone inhibited morphine-evoked mesolimbic dopamine release.*

We infused rats with saline or different doses of naltrexone (0.01, 0.1, 0.3 mg/kg, i.v.) before infusion of morphine (1 mg/kg, i.v.). For rats pretreated with saline, morphine increased dopamine (about 50% above baseline), and this increase was sustained throughout the time course analyzed (Figure 3.3). Naltrexone dose-dependently attenuated morphine-stimulated dopamine release in the NAcS. We found that, compared to saline, naltrexone caused a significant reduction in morphine-stimulated dialysate dopamine ($F_{21, 138} = 9.50$, $P < 0.05$, for the naltrexone dose x time interaction). Post hoc analyses revealed that at 5 min after morphine infusion, saline controls were different from all naltrexone pretreated rats. At all other time points afterwards, saline controls were different from naltrexone doses 0.1 and 0.3 mg/kg ($P < 0.05$) (Figure 3.3). Also, a difference between the effect of different doses of naltrexone on the AUC of morphine-evoked dopamine was detected ($F_{3, 23} = 55.87$, $P < 0.05$), and further analysis using Tukey HSD showed that naltrexone doses (mg/kg) 0.1 and 0.3, but not 0.01, are different from controls (Figure 3.4). Basal dopamine levels (nM)

(before morphine infusion) for saline and naltrexone doses 0.01, 0.1, and 0.3 mg/kg were 1.0 ± 0.3, 1.9 ± 0.4, 1.8 ± 0.5 and 1.2 ± 0.4 respectively (n = 5-7).

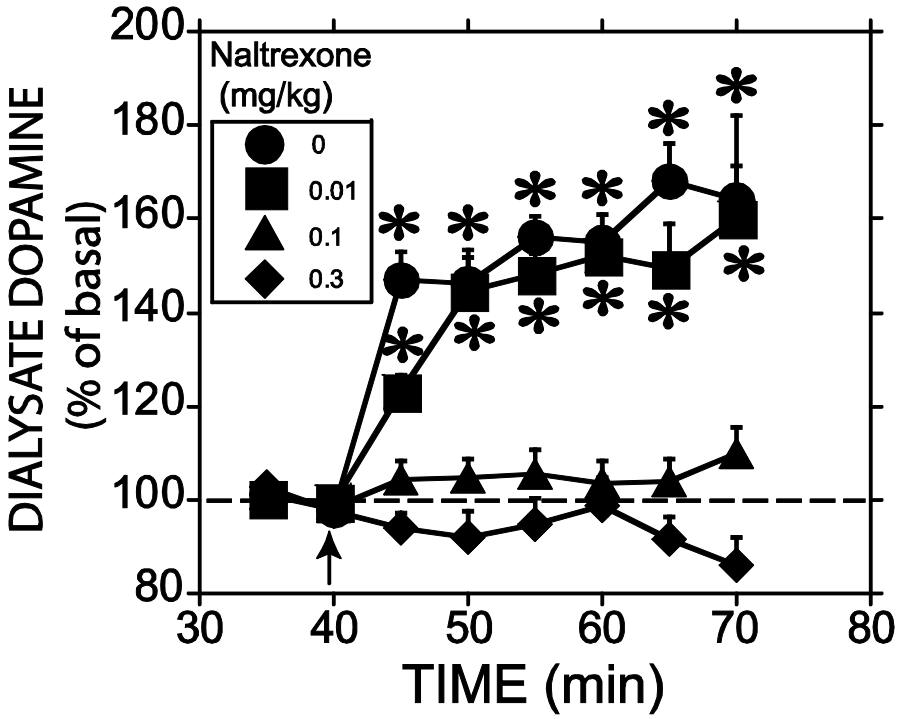



**Figure 3.3**. Naltrexone dose-dependently inhibits morphine-evoked dopamine release in the nucleus accumbens shell (time course). This shows the dopamine response in the shell of the nucleus accumbens of male Long Evans rats after i.v. pretreatment with saline (control) and naltrexone (0.01, 0.1, 0.3 mg/kg) and i.v. infusion with morphine (1 mg/kg). Each rat received an i.v. pretreatment of the different naltrexone doses followed 20 min later by morphine (1 mg/kg) (n = 5 - 7). Mean ± SEM are shown for each point. * shows a significant difference from basals of all doses. Arrow indicates the morphine infusion time.

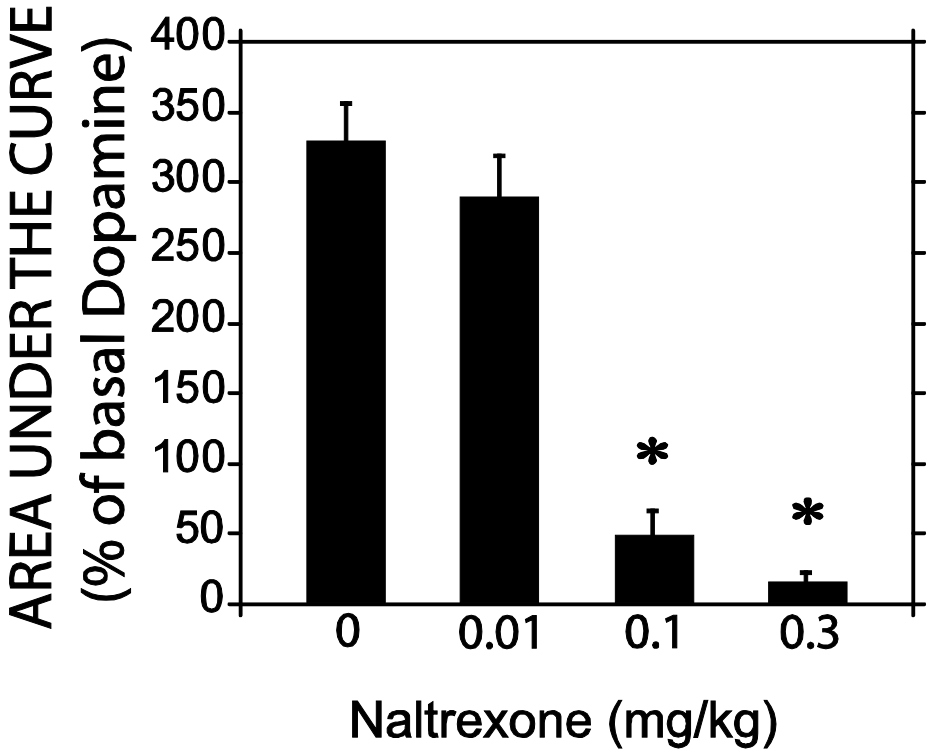



**Figure 3.4**. Naltrexone dose-dependently inhibits morphine-evoked dopamine release in the nucleus accumbens shell (AUC). This shows the AUC (% basal dopamine) in the nucleus accumbens shell after i.v. pretreatment with saline and naltrexone doses (0.01, 0.1, and 0.3 mg/kg) followed by i.v. infusion with morphine (1 mg/kg). * shows a significant difference from saline controls.

*Naltrexone attenuated ethanol-evoked mesolimbic dopamine release.*

For these experiments, we infused saline or naltrexone (0.3 or 1.0 mg/kg) 20 min before ethanol (10%w/v, 1 g/kg, i.v.) or saline (equivalent volume to ethanol) to determine the effect of naltrexone on ethanol-evoked dopamine release. For controls, ethanol increased dopamine (~33% from baseline), and this increase was sustained for at least 20 min (Figure 3.5), while saline did not cause an

increase in dopamine. ANOVA showed a naltrexone dose x time interaction ($F_{18, 292} = 1.67$, $P < 0.05$).

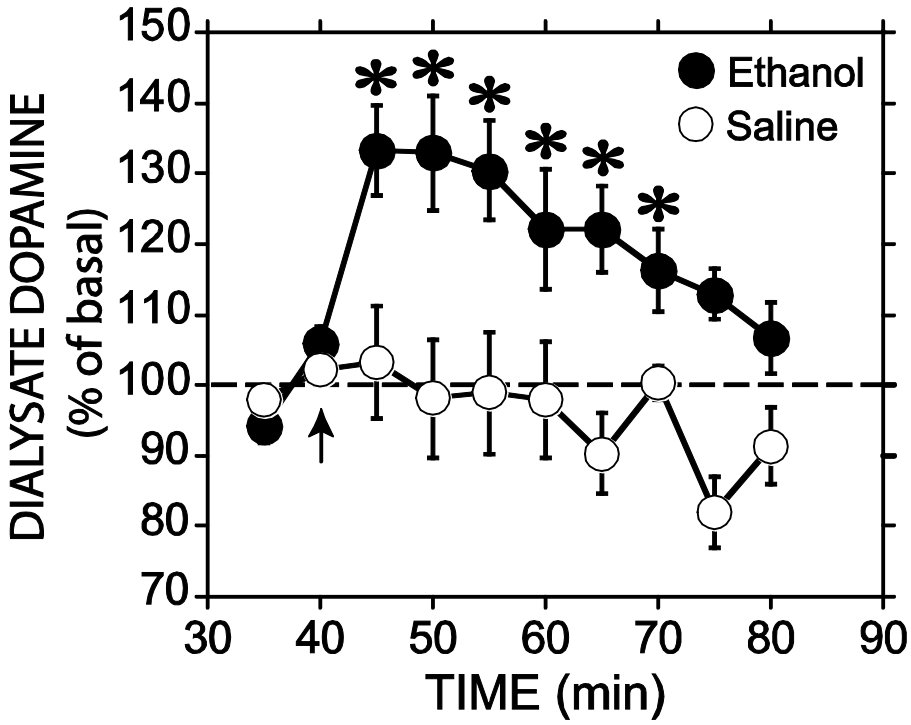



**Figure 3.5**. Ethanol, but not saline, increases dopamine in the nucleus accumbens shell. This shows dopamine response in the shell of the nucleus accumbens after i.v. pretreatment with saline and i.v. infusion with ethanol or saline. Each rat received an i.v. pretreatment of saline followed 20 min later by ethanol (1 g/kg, 10% w/v) (n = 8) or saline (n = 4) at the time indicated by the arrow. Mean ± SEM are shown for each point. * indicates significant difference from baseline. Volumes are 1 mL/ kg and 10 mL/ kg for naltrexone and ethanol/saline respectively. Arrow indicates the saline/ethanol infusion time.

For naltrexone pretreated rats, ethanol also increased dopamine (~24%), but this increase was not sustained beyond the first 5 min (Figures 3.6 and 3.7). ANOVA also revealed an ethanol x time interaction ($F_{9, 292} = 10.95$, $P < 0.05$), detecting that in all rats ethanol, but not saline, caused an increase in dopamine (Figure 3.8, saline data not shown). Post hoc analysis showed that compared to controls,

naltrexone prevents a prolongation of the increase in dopamine following ethanol administration (Figure 3.8). Naltrexone doses- 0.3 and 1.0 mg/kg do not dose-dependently attenuate ethanol-stimulated dopamine release. Though different from controls, ANOVA reveals that, with regards to the effect on ethanol-stimulated dopamine release, naltrexone 0.3 and 1.0 mg/kg are not different from each other (Figure 3.8).

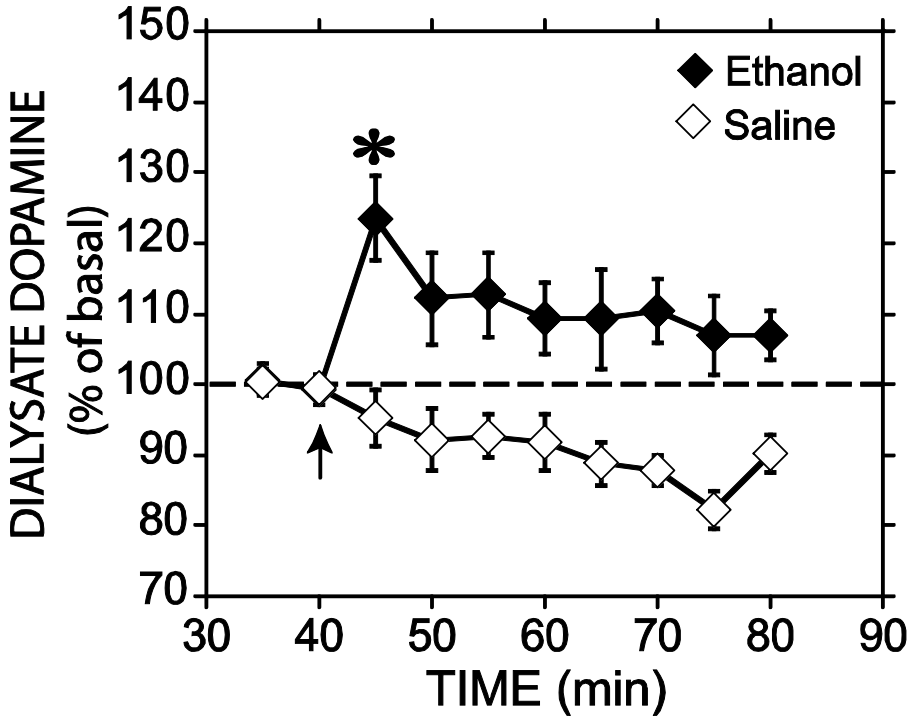



**Figure 3.6**. Naltrexone (0.3 mg/kg) attenuates the prolongation of ethanol-stimulated dopamine response. This shows dopamine response in the shell of the nucleus accumbens of male Long Evans rats after i.v. pretreatment with naltrexone (0.3 mg/kg) and i.v. infusion with ethanol or saline. Each rat received an i.v. pretreatment of naltrexone (0.3 mg/ kg) followed 20 min later by ethanol (1 g/kg, 10% w/v) (n = 8) or saline (n = 6). * indicates significant difference from baseline. Volumes are 1 mL/ kg and 10 mL/ kg for naltrexone and ethanol/saline respectively. Arrow indicates the saline/ethanol infusion time.

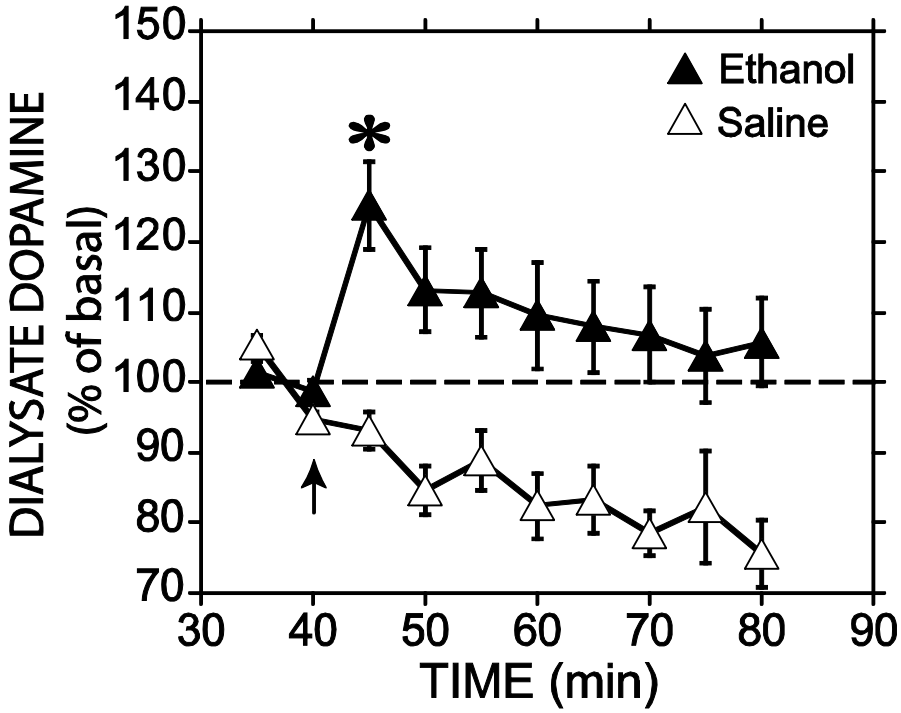



**Figure 3.7**. Naltrexone (1.0 mg/kg) attenuates the prolongation of ethanol-stimulated dopamine response. This shows dopamine response in the shell of the nucleus accumbens of male Long Evans rats after i.v. pretreatment with naltrexone (1.0 mg/kg) and i.v. infusion with ethanol or saline. Each rat received an i.v. pretreatment of naltrexone (1.0 mg/ kg) followed 20 min later by ethanol (1 g/kg, 10% w/v) (n = 8) or saline (n = 5). * indicates significant difference from baseline. Volumes are 1 mL/ kg and 10 mL/ kg for naltrexone and ethanol/saline respectively. Arrow indicates the saline/ethanol infusion time.

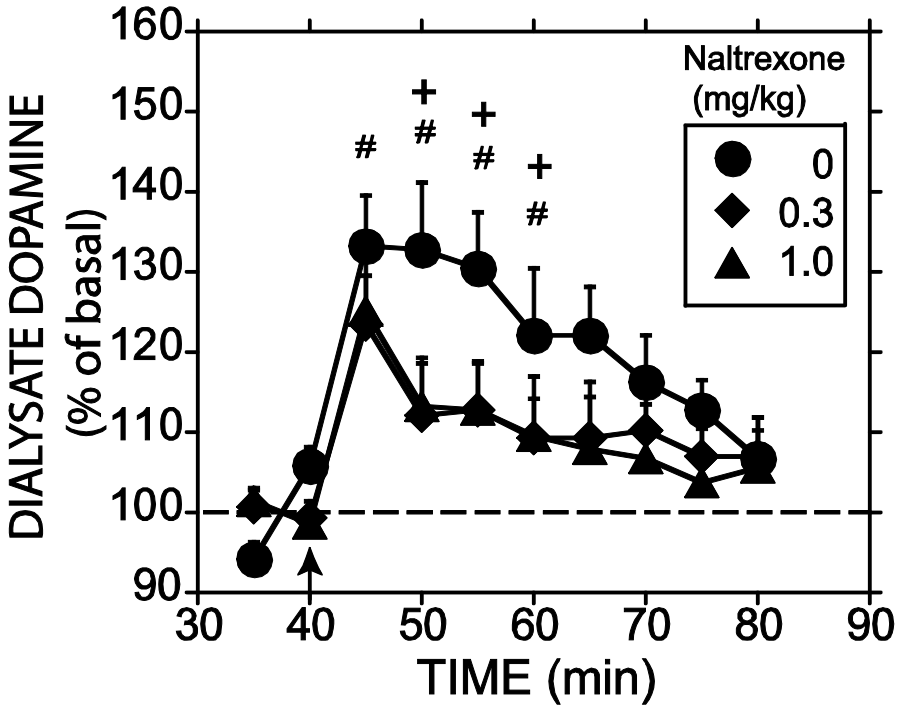



**Figure 3.8**. Naltrexone attenuates a delayed component of ethanol-stimulated dopamine response. This shows dopamine response after i.v. pretreatment with saline, naltrexone 0.3 and 1.0 mg/kg and i.v. infusion with ethanol (1 g/kg, 10% w/v). The data for control and each naltrexone dose is collapsed across all ethanol and saline infusions. Saline infusion does not change dialysate dopamine levels, and is therefore not shown. The analysis was restricted to first 20 min after infusion. The **#** and the **+** show significant difference between pretreatment with saline and naltrexone 0.3 mg/kg,and between saline and naltrexone 1.0 mg/kg, respectively.

It is important to add that ANOVA revealed that there was no effect of saline infusion on the control and naltrexone pretreated rats within the first 20 min after saline infusion, but afterwards there was a gradual decrease in basal dopamine (see Figures 3.5, 3.6, 3.7). Therefore, the analysis was restricted to the first 20 min after ethanol or saline infusion as shown in Figure 3.8. Basal dopamine concentrations (nM), before ethanol or saline infusions, for rats pretreated with saline and administered ethanol and saline were 1.0 ± 0.3 and 1.1 ± 0.3, respectively. For rats pretreated with naltrexone dose 0.3 mg/kg and infused with

saline or ethanol, the basal dopamine levels (nM) were 0.9 ± 0.2 and 1.2 ± 0.1, respectively. For naltrexone 1.0 mg/kg pretreated rats, the basal levels (nM) were 1.2 ± 0.4 and 1.3 ± 0.1 for saline and ethanol, respectively.

Naltrexone did not alter peak ethanol concentration and time course. The peak ethanol concentrations were 5.9 ± 0.4, 6.5 ± 0.5 and 6.3 ± 0.6 mM for control, naltrexone 0.3 and 1.0 mg/kg (n = 8 each) pretreated rats, respectively. There was no significant difference in the peak ethanol concentration ($F_{2, 23} = 0.40$, $P > 0.05$, for the naltrexone dose x time interaction) and in the time course of ethanol ($F_{11, 231} = 0.62$, $P > 0.05$, for the naltrexone dose x time interaction) (Figure 3.9).

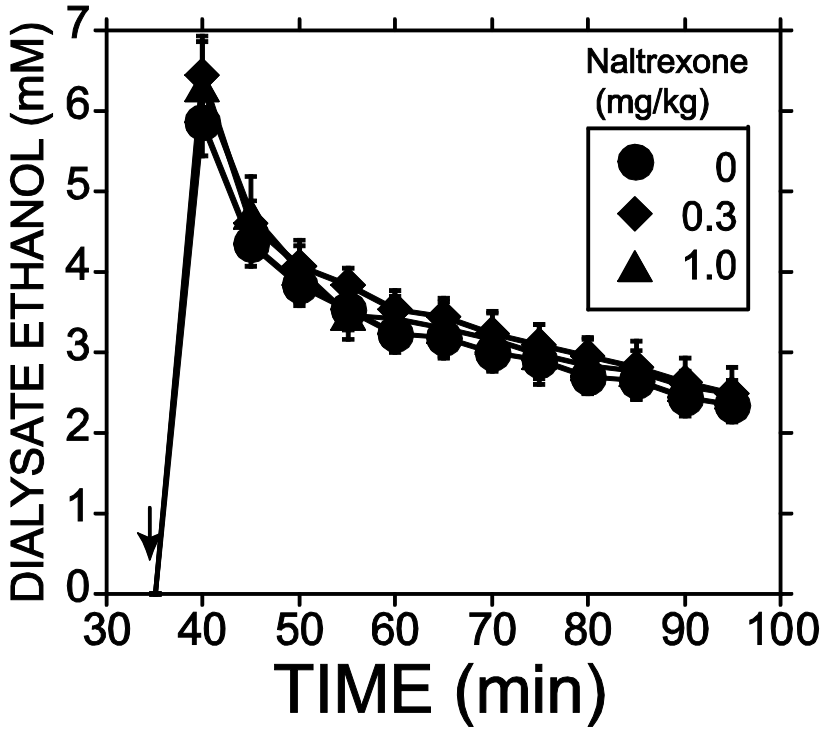



**Figure 3.9**. Naltrexone does not affect the peak and time course of ethanol in the nucleus accumbens shell. This shows ethanol concentrations in the shell of the nucleus accumbens of rats after i.v. pretreatment with saline and naltrexone (0.3 and 1 mg/kg) and i.v. infusion with ethanol (1 g/kg, 10% w/v). Mean ± SEM are shown for each point. All groups have n = 8. Arrow indicates the ethanol infusion time.

*β-funaltrexamine inhibited morphine-evoked mesolimbic dopamine release.*

In these experiments, we injected saline or β-funaltrexamine (20 mg/kg, s.c.) followed 20-24 h later by saline and morphine (1 mg/kg, i.v.) to determine the effect of β-funaltrexamine on morphine-induced dopamine release in the NAcS. Morphine (but not saline) increased the release of dopamine in the NAcS (Figures 3.10 and 3.11).

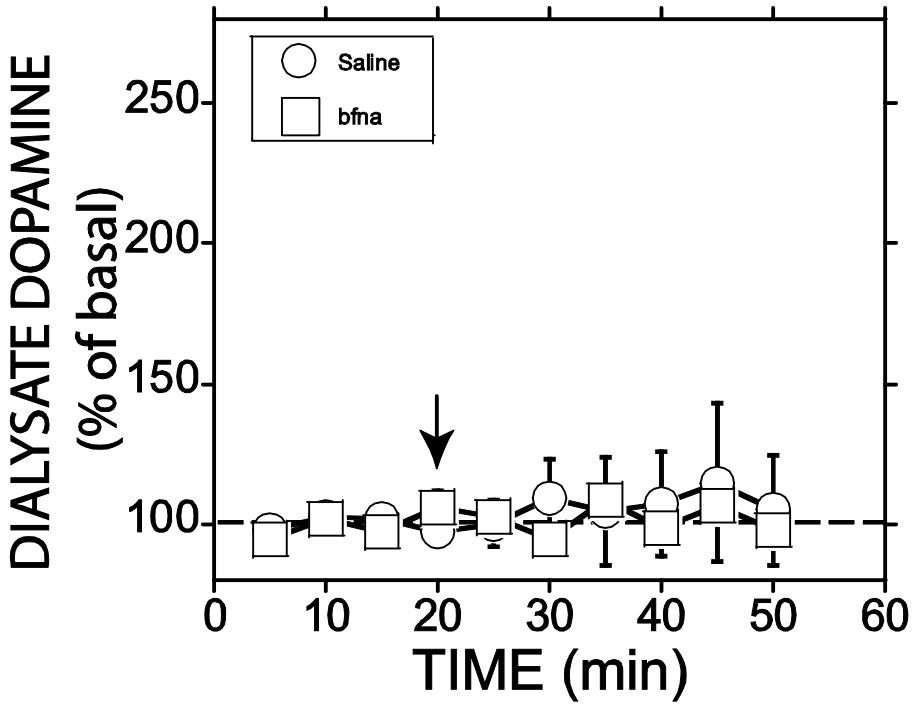



**Figure 3.10**. Saline does not increase dopamine in control and β-funaltrexamine pretreated rats. This is the dopamine response in the shell of the nucleus accumbens of rats after s.c. pretreatment with saline (n = 5) and β-funaltrexamine (20 mg/kg) (n = 6) followed 20-21 h later by i.v. infusion with saline. The volume of saline infused = 1 mL/kg.

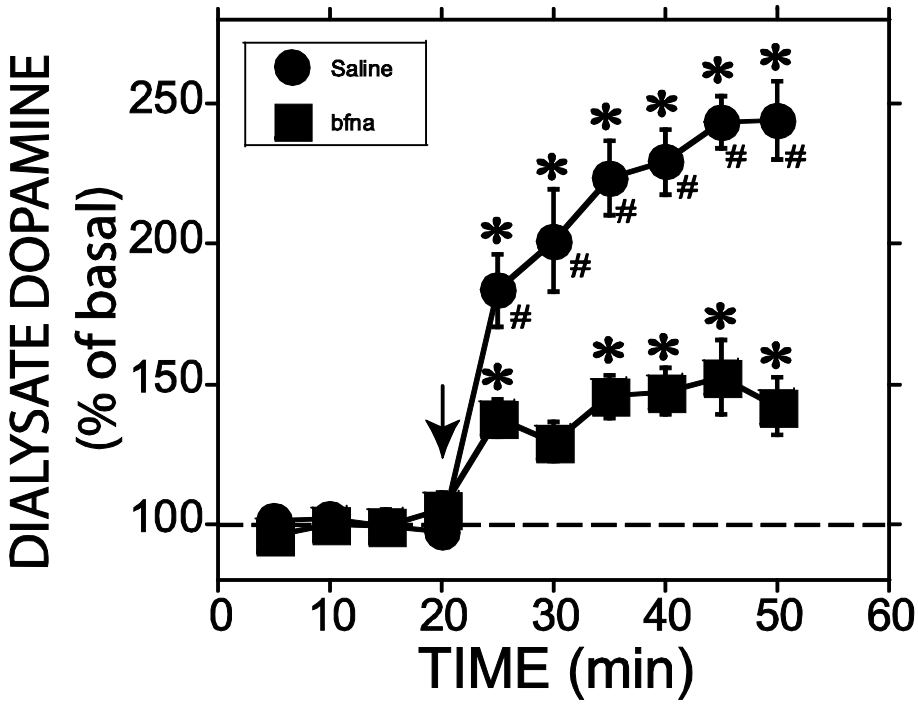



**Figure 3.11**. β-funaltrexamine attenuates morphine-evoked dopamine release in the nucleus accumbens shell in rats (time course). This is the dopamine response in the shell of the nucleus accumbens of rats after s.c. pretreatment with saline (control) and β-funaltrexamine (20 mg/kg) followed 24 h later by i.v. infusion with morphine (1 mg/kg) (n = 6 each). Arrow indicates the morphine infusion time. * indicates significant difference from baseline. The **#** shows significant difference between control and β-funaltrexamine (20 mg/kg), comparing individual time points after the infusion.

The basal dopamine levels, before morphine administration, were 1.2 ± 0.3 and 1.6 ± 0.4 nM for control and rats pretreated with β-funaltrexamine (20 mg/kg, s.c.), respectively (n = 6 each). For β-funaltrexamine pretreated rats, the increase in dopamine after morphine was significantly attenuated compared to controls (Figure 3.11). ANOVA showed an effect of time ($F_{9, 90} = 56.51$, $P < 0.05$) and a β-funaltrexamine x time interaction ($F_{9, 90} = 13.74$, $P < 0.05$). Post hoc analysis showed that controls are different from the β-funaltrexamine treated animals at every time point after morphine infusion ($P < 0.05$) (Figure 3.11). Compared to morphine, saline does not increase dopamine in control (n =5) and β-

funaltrexamine (20 mg/kg s.c.) (n = 6) pretreated rats ($F_{9, 79} = 0.33$, $P > 0.05$, for the β-funaltrexamine x time interaction) (Figure 3.10). Also, a One-way ANOVA analysis of the morphine-evoked dopamine AUC showed that there was a difference between controls (n = 6) and β-funaltrexamine (n = 6) ($F_{1, 11} = 47.12$, $P < 0.05$) (Figure 3.12).

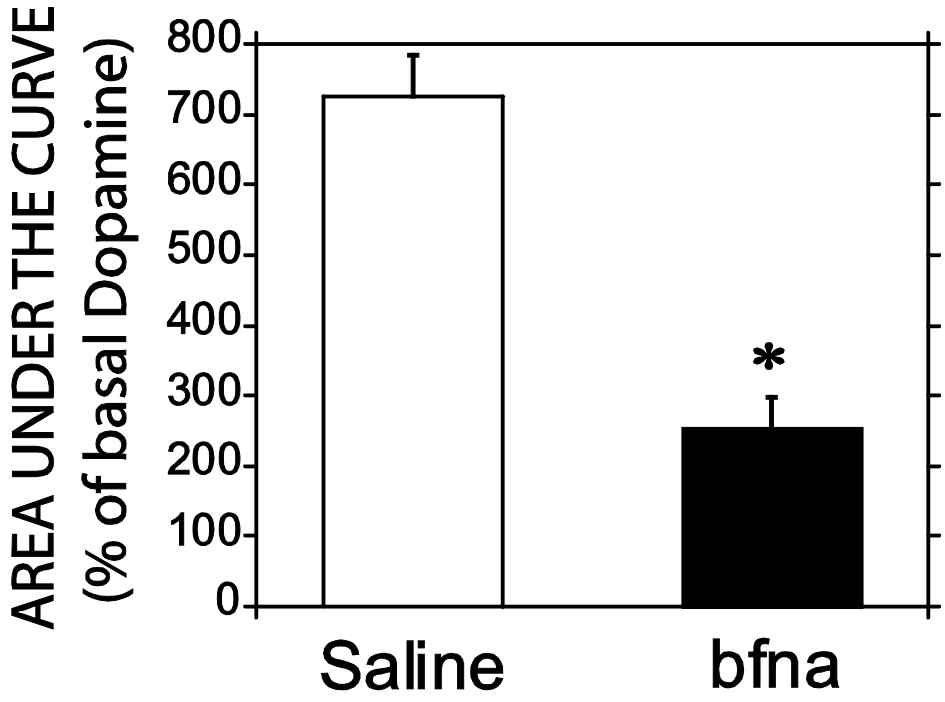



**Figure 3.12**. β-funaltrexamine inhibits morphine-evoked dopamine release in the nucleus accumbens shell (AUC). This shows the AUC (% basal dopamine) in the nucleus accumbens shell after i.v. pretreatment with saline and β-funaltrexamine (20 mg/kg) followed 20-24 h later by i.v. infusion with morphine (1 mg/kg). * shows a significant difference from saline controls. bfna = β-funaltrexamine.

*β-funaltrexamine attenuated ethanol-evoked mesolimbic dopamine release.*

In these experiments, rats were pretreated subcutaneously with saline (control) or β-funaltrexamine (20 mg/kg), followed 20-25 h by saline and ethanol (10%w/v, 1 g/kg, i.v.). The saline i.v. treatment was equivalent to the ethanol volume, and all control and β-funaltrexamine pretreated animals received saline followed by ethanol infusions. The control (n = 8) and β-funaltrexamine groups (n = 6) had basal dopamine concentrations (before ethanol infusion) of 1.2 ± 0.2 and 1.0 ± 0.2 nM respectively. For control rats, ethanol, but not saline, increased dopamine (~50% of baseline) (Figure 3.13 and 3.14). We determined that β-funaltrexamine

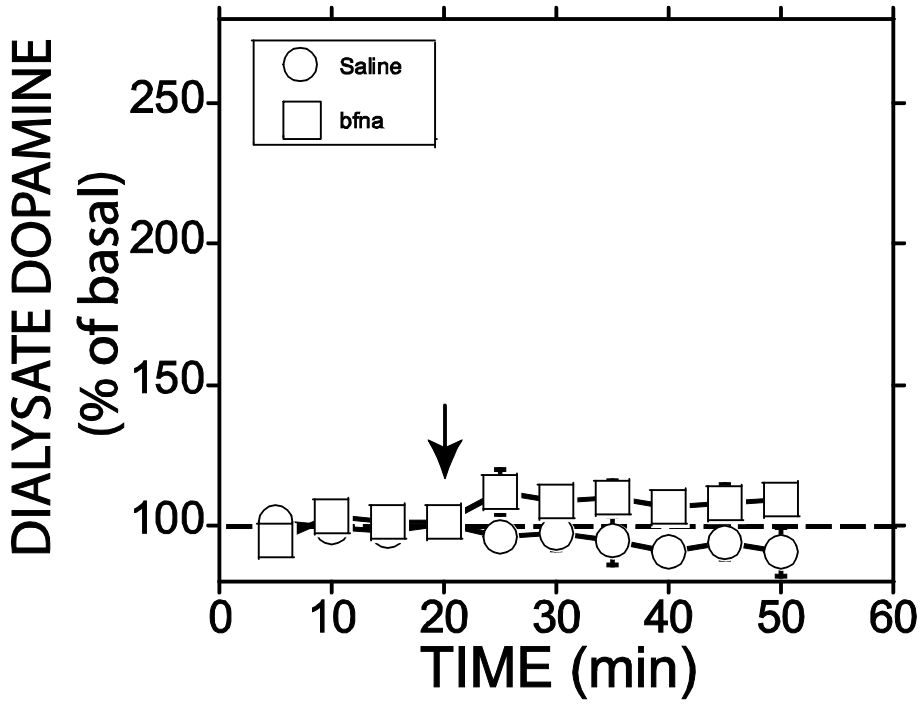



**Figure 3.13**. β-funaltrexamine does not change the effect of saline infusion on dopamine release in the nucleus accumbens shell. This is the dopamine response in the shell of the nucleus accumbens of rats after s.c. pretreatment with saline (n = 5) and β-funaltrexamine (20 mg/kg) (n = 6) followed 20-23 h later by i.v. infusion with saline. The volume of saline infused = 10 mL/kg.

attenuated ethanol-evoked mesolimbic dopamine release (Figure 3.14). ANOVA

detected significance as follows: ($F_{7, 82}$ = 19.86, P < 0.05, time), ($F_{7, 82}$ = 2.67, P < 0.05, for the β-funaltrexamine x time interaction).

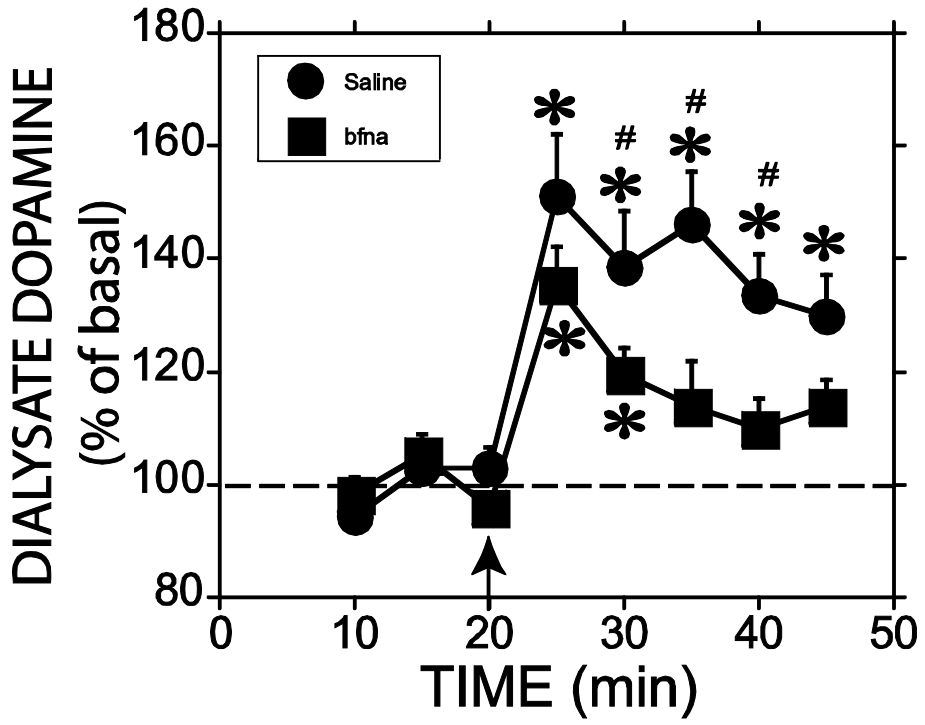



**Figure 3.14**. β-funaltrexamine attenuates ethanol-stimulated dopamine release in the nucleus accumbens shell. This is the dopamine response in the shell of the nucleus accumbens of rats after s.c. pretreatment with saline (n = 8) and β-funaltrexamine (20 mg/kg) (n = 6) followed 24-25 h later by i.v. infusion with ethanol (1 g/kg, 10% w/v). Arrow indicates the ethanol infusion time. The * shows significance compared to basals and the **#** shows significant difference between control and β-funaltrexamine pretreated rats, comparing individual time points after the infusion.

Post hoc analysis showed that compared to basals, control animals showed an increase in dopamine after ethanol for every time point (25 min after infusion) included in the analysis whereas β-funaltrexamine treated animals showed an increase in dopamine only in the first 2 time points (10 min after infusion) (Figure 3.14). Compared to ethanol, saline does not cause any change in dopamine

levels in β-funaltrexamine and control animals (n = 5 each) (Figure 3.13). There was no significant difference in dialysate dopamine from the NAcS after saline was administered intravenously to control and β-funaltrexamine (20 mg/kg, s.c.) pretreated rats ($F_{9, 69} = 1.99$, $P > 0.05$, for the β-funaltrexamine x time interaction) (Figure 3.13).

β-funaltrexamine did not alter dialysate ethanol peak and time course. The peak ethanol concentrations were 5.5 ± 0.8 and 5.9 ± 0.5 mM for control (n = 8) and β-funaltrexamine (20 mg/kg) (n = 6) pretreated rats respectively, and there was no significant difference in the peak ethanol concentration ($F_{1, 13} = 0.17$, $P > 0.05$, for the β-funaltrexamine x time interaction) and in the time course of ethanol ($F_{11, 132} = 0.30$, $P > 0.05$, for the β-funaltrexamine x time interaction) (Figure 3.15).

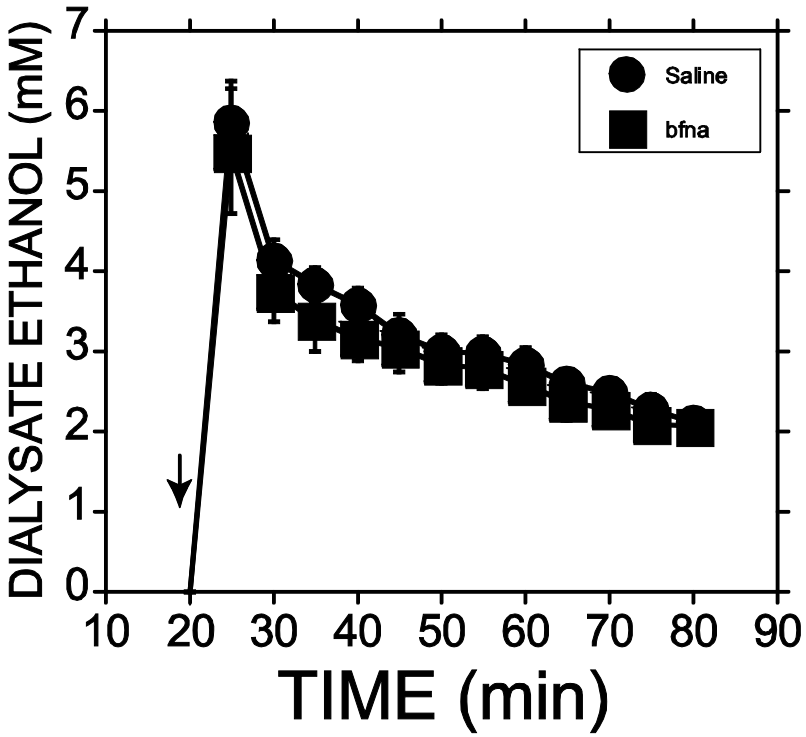



**Figure 3.15**. β-funaltrexamine does not affect the peak concentration and time course of ethanol in the nucleus accumbens shell. This shows the ethanol concentrations in the shell of the nucleus accumbens of male Long Evans rats after i.v. pretreatment with saline (n = 8) and β-funaltrexamine (bfna) (20 mg/kg) (n = 6) followed 24-25 h later by i.v. infusion with ethanol (1 g/kg, 10% w/v). Mean ± SEM are shown for each point. Arrow indicates the ethanol infusion time.

## Discussion

The hypothesis that the μ-opioid receptors play a role in the mechanism of ethanol-stimulated dopamine in the NAcS is supported by this data. We show that naltrexone, a non-selective opioid antagonist, did not affect basal dopamine levels (Figure 3.2), but dose-dependently attenuated morphine-evoked dopamine release in the NAcS (Figures 3.3 and 3.4). At doses effective in attenuating

morphine-evoked dopamine release in the NAcS, naltrexone did not prevent ethanol from increasing dopamine, but instead prevented the dopamine increase from being prolonged (Figure 3.8). β-funaltrexamine, a selective irreversible μ-opioid antagonist (Ward et al., 1982, 1985; Liu-Chen and Phillips, 1987; Liu-Chen et al., 1990, 1991), at a dose that significantly attenuated morphine-evoked dopamine release in the NAcS (Figures 3.11 and 3.12), also inhibited the prolongation of ethanol-evoked dopamine release (Figure 3.14). Neither naltrexone nor β-funaltrexamine affected the pharmacokinetics of ethanol in the brain (Figures 3.9 and 3.15). However, we detected a gradual decrease in dopamine levels beginning at 20 min after saline administration (Figure 3.5, 3.6 and 3.7). Therefore, we restricted our analysis to first 20 min after ethanol and saline infusions. We cannot explain this gradual decrease in basal dopamine after naltrexone pretreatment and saline infusion. However, we do know that this is not due to naltrexone infusion, because naltrexone does not change basal dopamine levels (Figure 3.2). Notwithstanding this potential confound to the interpretation of our naltrexone data, we obtained a similar effect with β-funaltrexamine on ethanol-stimulated dopamine release in the NAcS, reinforcing our conclusions. Together, our data support our conclusion that the μ-opioid receptors are involved in a delayed component of the mechanism of ethanol-stimulated dopamine release in the NAcS of ethanol-naïve rats.

The NAc is divided into core and shell sub-regions. We sampled from the shell in all rats used in our experiments. Compared to the NAc core, the NAc shell is more sensitive to the dopamine-stimulating effects of morphine and ethanol (Pontieri et al., 1995; Howard et al., 2008). There are multiple opioid receptors (μ,

δ, and κ), and activation of μ- and δ-opioid receptors enhances mesolimbic dopamine release (for review, see Herz, 1997). In order to characterize the specific contribution of the μ-opioid receptor in ethanol-stimulated dopamine release, we used morphine (selective systemic μ-opioid receptor agonist), as a positive control to determine the doses of naltrexone and β-funaltrexamine that are effective in attenuating morphine-evoked dopamine release in the NAcS (Figures 3.3, 3.4, 3.11 and 3.12). To our knowledge, we are the first to do a dose-response study involving the effect of naltrexone on morphine-evoked mesolimbic dopamine. We are also the first to study the effect of systemic β-funaltrexamine on morphine-induced dopamine release in the NAc. Morphine increases mesolimbic dopamine release, and though morphine has been shown to act at the different opioid receptors (including μ- and δ-opioid receptors which activate mesolimbic dopamine), the morphine-evoked increase in mesolimbic dopamine is predominantly due to activation of the μ-opioid receptor. For instance, both intracerebroventricular administered β-funaltrexamine (Di Chiara and Imperato, 1988b) and systemic naloxone (Borg and Taylor, 1997) suppressed systemic morphine-evoked dopamine increase in the NAc, whereas systemic naltrindole, a highly selective δ-opioid antagonist, did not (Borg and Taylor, 1997). Moreover, μ-opioid receptor knockout mice showed attenuation in mesolimbic dopamine release due to experimenter-administered morphine (Chefer et al., 2003) whereas δ-opioid receptor knockout mice did not. In addition, morphine has a significantly higher (> 100 fold) affinity for the μ- relative to the δ-opioid receptor (Raynor et al., 1994). Thus, systemic morphine is predominantly acting at the μ-receptors to increase dopamine in the NAc. This makes morphine a good positive control. Our data shows that naltrexone

(Figures 3.3 and 3.4) and β-funaltrexamine (Figures 3.11 and 3.12), through blockade of the μ-opioid receptor, suppressed morphine-evoked dopamine release in the NAcS. A limitation of the naltrexone experiments is that the non-selective nature of naltrexone makes conclusions regarding specific opioid receptors difficult to make. This limitation is addressed by the experiments with β-funaltrexamine-a selective μ-opioid receptor antagonist. However, a limitation of the β-funaltrexamine experiments is that β-funaltrexamine also binds to the μ-δ opioid receptor complex (Rothman et al., 1988, 1991), and we cannot rule this out.

The mechanism of ethanol-evoked mesolimbic dopamine is thought to include ethanol-stimulated endogenous opioid release to activate the μ-opioid receptors (Herz, 1997; Xiao and Ye, 2008). Our data shows that naltrexone (Figure 3.8) and β-funaltrexamine (Figure 3.14), at doses effective in suppressing morphine-evoked dopamine release in the NAcS (Figures 3.3, 3.4, 3.11, and 3.12), attenuated ethanol-induced dopamine release. To the best of our knowledge, we are the first to study the effect of systemic β-funaltrexamine on ethanol-induced dopamine release in the NAc.

Our naltrexone data (Figure 3.8) supports another publication that shows that naltrexone attenuates ethanol-stimulated mesolimbic dopamine release. Benjamin et al. (1993) reported that naltrexone reverses ethanol-induced dopamine release in the NAc in awake, freely moving Long Evans rats. However, some limitations inherent in the experimental design by Benjamin et al. (1993) include continuous local delivery into the NAc of a very high dose of ethanol (>

800 mM) throughout the duration of the experiment (200 min). We reached the same conclusions reached by Benjamin et al. (1993), though we employed systemic administration of ethanol, presenting a more comprehensive and realistic view of the effect of naltrexone on acute ethanol-evoked mesolimbic dopamine release.

Our data shows that the attenuating effects on the ethanol-stimulated dopamine response of both naltrexone and β-funaltrexamine are not immediate but delayed (Figures 3.8 and 3.14). We were able to detect this delay in the μ-opioid receptor-mediated component of the ethanol-evoked dopamine response because in our experiments, we analyzed changes in dopamine in the NAc over relatively short time periods (5 min).This presents a better time resolution of the changes in dopamine compared to other similar microdialysis studies (20 min) that have attempted to characterize the role of the opioid receptors in the mechanism of ethanol-stimulated mesolimbic dopamine release (Acquas et al., 1993; Benjamin et al., 1993; Tanda and Di Chiara, 1998).

There are other studies that show that the μ-opioid receptor component in the mechanism of ethanol-stimulated dopamine release is delayed. Naloxonazine, an irreversible selective μ1-opioid receptor subtype antagonist, blocks morphine and ethanol-mediated dopamine release in the NAc (Tanda and Di Chiara, 1998) in freely-moving male Sprague-Dawley rats. After careful study of the data in the paper by Tanda and Di Chiara (1998), we realize that, similar to our results, the effect of μ1-opioid receptor blockade on the dopamine response due to ethanol (0.25 and 0.5 g/kg i.p.) was delayed (see Tanda and Di Chiara, 1998). In

addition, we published our findings of the effect of ethanol on dopamine release in the ventral striatum of C57BL/6 mice (Job et al., 2007), and determined that the μ-opioid receptor is involved in this mechanism. However, the data (see Job et al., 2007) shows that μ-opioid knockout males on the mixed genetic background (C57BL/6J-129SvEv) showed increases in dopamine evoked by 2 g/kg ethanol i.p. injection (similar to their wildtype controls), but this dopamine increase returned to baseline more rapidly compared with controls. It is important to note that μ-opioid knockout females (unlike the males) on the mixed genetic background (C57BL/6J- 129SvEv), and μ-opioid knockouts on the congenic C57BL/6J background, showed a complete blockade of ethanol-stimulated dopamine release (Job et al., 2007).

Thus, our present data together with previous findings (Benjamin et al., 1993; Tanda and Di Chiara, 1998; Job et al., 2007) all reinforce the idea that the μ-opioid receptor is involved in a delayed component of the ethanol-stimulated mesolimbic dopamine response. We do not know why there is a delay in the opioid effect on ethanol-induced mesolimbic dopamine response (Figures 3.8 and 3.14), and further research needs to be done clarify this. However, ethanol can increase dopamine through multiple non-opioid mechanisms, and these other mechanisms may be involved in the early component of the dopamine response.

In conclusion, we have presented data showing that the μ-opioid receptors are involved in ethanol-stimulated dopamine in the NAcS of ethanol-naïve rats. Furthermore, the μ-opioid receptors are involved in a delayed component of the

mechanism of ethanol-stimulated dopamine release in the NAcS of ethanol-naïve rats. It is important to add that our conclusions are limited to the NAcS as we did not determine the effects of μ-opioid receptor blockade on the mechanism of ethanol-evoked dopamine release in other regions of the NAc.

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# Chapter 4. Summary and Conclusions

The overall hypothesis for the dissertation is that the μ-opioid receptors play a role in the mechanism of ethanol-stimulated mesolimbic dopamine release. The experimental techniques included jugular catheterizations and stereotaxic surgeries that were done to prepare the rats for intravenous drug administration and microdialysis, respectively. Microdialysis sampling time was 5 min, and dialysate samples were analyzed for ethanol and dopamine by GC and HPLC, respectively. We did histological analysis to determine that the dialysis probes had been placed into the nucleus accumbens shell (NAcS). The experiments were designed to determine the effect of μ-opioid antagonists on morphine- and ethanol-stimulated mesolimbic dopamine release. We did two sets of experiments - the naltrexone and the β-funaltrexamine experiments. There are no literature reports looking at naltrexone dose-response effect on morphine-induced dopamine release. In addition, the only record in the literature for the effects of naltrexone on acute ethanol-induced mesolimbic dopamine release involved a local administration of ethanol into the NAc (Benjamin et al., 1993). We present a more comprehensive design and more thorough analysis of naltrexone using systemic ethanol administration. A previous experiment had looked at the effect of intracerebroventricularly administered β-funaltrexamine on morphine-induced dopamine release (Di Chiara and Imperato, 1988b), but we are the first to report on the effect of systemic β-funaltrexamine on morphine-induced dopamine release in the NAcS. We are also the first to report on the effect of systemic β-funaltrexamine on ethanol-induced dopamine release in the NAcS.

For the naltrexone experiments, we did a dose-response study in order to determine naltrexone doses that were effective in attenuating morphine-induced dopamine release in the NAcS. This is a positive control experiment, and morphine was used because it is a relatively selective μ-opioid agonist. Naltrexone is a non-selective opioid antagonist, and we tried to determine a dose that achieved a significant blockade of the μ-opioid receptors involved in mesolimbic dopamine release. We found that morphine, via activation of the μ-opioid receptors, increases dopamine in the NAcS. This is supported by evidence in the literature showing that the μ-opioid receptors play a role in morphine-evoked mesolimbic dopamine release. Furthermore, we determined that naltrexone dose-dependently attenuates this morphine-evoked dopamine release. We inferred that at the doses effective in suppressing morphine-evoked dopamine release, naltrexone achieves a significant blockade of the μ-opioid receptors that are involved in mesolimbic dopamine release.

Another set of experiments was done to determine if naltrexone, at doses effective in suppressing morphine-evoked dopamine release, suppresses ethanol-stimulated dopamine release in the NAcS. We determined that naltrexone was effective in attenuating ethanol-stimulated dopamine release in the NAcS. However, we noticed that this attenuation was not immediate. Further analysis found that the ethanol-induced dopamine response consisted of two components – an early non-opioid component that was not responsive to naltrexone, and a delayed opioid component that was responsive to naltrexone. We also noticed that there was no dose-dependent effect of increasing the naltrexone dose. We concluded that naltrexone attenuates ethanol-stimulated dopamine release, though this effect is delayed. We concluded that the μ-opioid

receptors are involved in a delayed component of ethanol-stimulated dopamine release in the NAcS in naïve rats.

As we mentioned in the previous paragraph, there is no dose-dependent effect of naltrexone on ethanol-stimulated dopamine release beyond a naltrexone dose of 0.3 mg/kg. Of the opioid receptors, naltrexone has the highest affinity at μ-opioid receptors, and increasing the dose therefore is presumed to cause additional blockade of other opioid receptors including the δ-opioid receptor. However, this did not cause an additional inhibition of ethanol-stimulated dopamine release. We speculate that δ-opioid receptors contribute to ethanol-stimulated dopamine release via interaction with the μ-opioid receptors (see more details in section of general introduction regarding μ- and δ-opioid receptor interactions in the mechanism of mesolimbic dopamine release). Therefore when significant populations of the μ-opioid receptors that are involved in mesolimbic dopamine release have been blocked, they are not available to interact with δ-opioid receptors, and activation of additional δ-opioid receptors may not lead to an increase in mesolimbic dopamine release. A limitation of this experiment is that even though we used naltrexone doses $\leq$ 1 mg/kg, we cannot rule out the involvement of other opioid receptors in the effects of naltrexone. We decided to use a more selective μ-opioid receptor antagonist as a tool to further strengthen our conclusions.

There is extensive evidence that β-funaltrexamine is a selective irreversible μ-opioid receptor antagonist (Ward et al., 1982, 1985; Liu-Chen and Phillips, 1987; Liu-Chen et al., 1990, 1991). For the β-funaltrexamine experiments, like the naltrexone experiments, we determined a dose of β-funaltrexamine that was effective in significantly attenuating the morphine-evoked dopamine release in

the nucleus accumbens shell (positive control). The effective dose of β-funaltrexamine is thought to achieve a significant blockade of the μ-opioid receptors in order to attenuate morphine-induced dopamine release. Our data show that β-funaltrexamine, at doses effective in suppressing morphine-evoked dopamine release in the nucleus accumbens shell, attenuated ethanol-induced dopamine release. The results from the β-funaltrexamine experiments reinforce our conclusions from the naltrexone experiments. We have confirmed that the μ-opioid receptors play a role in the mechanism of ethanol-stimulated dopamine release. However, again, the opioid-driven component of the ethanol-stimulated dopamine response is delayed.

Our results support the overall hypothesis of this dissertation. However, we have been able to uncover for the first time a delay in the μ-opioid effects. We were able to detect this delay in the μ-opioid receptor-mediated component of the ethanol-evoked dopamine response because in our experiments, we analyzed changes in dopamine in the NAc over relatively short time periods (5 min).This presents a better time resolution of the changes in dopamine compared to other similar microdialysis studies (20 min) that attempt to characterize the role of the opioid receptors in the mechanism of ethanol-stimulated mesolimbic dopamine release (Acquas et al., 1993; Benjamin et al., 1993; Tanda and Di Chiara, 1998). This is a significant contribution to the field. However, this finding raises more questions about the opioid-driven mechanism of ethanol-stimulated dopamine release. For example: Why is the μ-opioid effect delayed? What non-opioid mechanisms constitute the early component of the dopamine response? How may this finding be related to ethanol reinforcement and even the pharmacotherapy of ethanol dependence?

Why is the μ-opioid effect delayed? We propose that two broad mechanisms may be involved in the opioid effect on ethanol-stimulated dopamine. The opioids could be prolonging the dopamine release by either increasing the dopamine release, or by preventing its decrease. Since opioids are known to be inhibitory, it is likely that the prominent mechanism here will be a prevention of the decrease in dopamine. This can be done by opioid-mediated inhibition of mechanisms that would otherwise have decreased the dopamine release. Such inhibitory mechanisms could be exerted by GABA or glycine. Apart from these, it is tempting to speculate that the opioids may have some inhibitory effect at monoamine transporters such as the dopamine transporter, but there is no evidence of this in the literature. Thus, we are left with the idea that opioids inhibit GABAergic neurons, preventing the decrease in dopamine release, in order to prolong the effect of ethanol-stimulated dopamine release. However, the question as to the mechanism still remains.

More acceptable ideas about the opioid mechanisms of ethanol-stimulated mesolimbic dopamine release have to do with ethanol-stimulated release of the endogenous opioid peptides. It has been proposed that one of the mechanisms by which ethanol stimulates dopamine release in the NAcS is by increasing β-endorphin release into the VTA. Dopamine neurons in the VTA receive inhibitory inputs from GABAergic afferents and interneurons, and the released β-endorphin is then proposed to act on μ-opioid receptors on the GABAergic neurons, inhibiting them, and thereby increasing VTA dopaminergic activity via disinhibition mechanisms (Herz, 1997). In the literature, however, there is no direct evidence showing that β-endorphin fibers from the hypothalamus project to the VTA (Khatchaturian and Watson, 1982). Also, evidence seems to suggest that the NAc is more sensitive than the VTA to the effects of β-endorphin

(Ableiter and Schulz, 1992), and this is buttressed by the observation that there are direct β-endorphin projections from the hypothalamus to the NAc and not the VTA (Khatchaturian and Watson, 1982). However, in the NAc, β-endorphin release seems to be a consequence and not the cause of dopamine release (Roth-Deri et al., 2003). One possible explanation is that β-endorphin is released in the VTA (Jarjour and Gianoulakis, 2006) or at other neuroanatomical structures and diffuses to access sites in the VTA. This may explain the delay in the opioid effects (see Figure 2.4). We know that β-endorphin is released by ethanol and involved in ethanol reinforcement. We are not very convinced, however, that it plays a role in the mechanism of ethanol-stimulated dopamine release. More investigations need to be done to clarify this.

To us, a more reasonable idea seems to be the involvement of enkephalins in the mechanism of ethanol-stimulated dopamine release. There are many instances in the literature that show that enkephalinergic neurons interact very extensively with the mesolimbic dopaminergic neurons. For instance, enkephalinergic cell bodies are found in the VTA (Finley et al., 1981b; Harlan et al., 1987), and enkephalinergic neurons synapse on the dopaminergic neurons and GABAergic interneurons in the VTA (Sesack and Pickel, 1992, 1995). Also, enkephalins have also been shown to be involved in dopaminergic tone, since inhibiting the metabolism of enkephalin in the ventral tegmental area leads to an increase in dopamine release in the NAc (Daugé et al., 1992). Enkephalins have also been shown to be co-localized in GABAergic neurons in the VTA and NAc (Sesack and Pickel, 1992, 1995; Kalivas et al., 1993; Curran and Watson, 1995). Furthermore, enkephalins have a high affinity and efficacy at the μ-opioid receptors (Raynor et al., 1994).

We propose that in the fast non-opioid component of the dopamine response, ethanol directly activates the dopaminergic neurons to increase dopamine. Also within this early component of the dopamine response, ethanol recruits some other non-opioid mechanisms such as serotonergic, acetylcholine, glycinergic and GABA. Ethanol also releases enkephalin (Marinelli et al., 2005). However, we propose that, as with other neuropeptides, enkephalin is not released at the synaptic sites, and has to diffuse some distance to access its postsynaptic target sites (see figure 2.4). This may account for the delay in the opioid effect. Enkephalin then inhibits the inhibitory GABA inputs (from afferents or interneurons), leading to an increase in dopaminergic neuron firing and increase in accumbal dopamine release.

We looked at the effect of μ-opioid receptors, and more likely candidates are the very highly selective endomorphins. Endomorphins project to the ventral tegmental area and the nucleus accumbens, and increase mesolimbic dopamine. However, in literature, the involvement of the endomorphins in the mechanism of ethanol-stimulated mesolimbic dopamine has not been determined. More research needs to be done to find out if endomorphins play a role in this mechanism.

How may the finding that the μ-opioid component of ethanol-stimulated dopamine response is delayed be related to ethanol reinforcement and even the pharmacotherapy of ethanol dependence? Naltrexone is thought to exert its suppressive effect on ethanol reinforcement through blockade of the μ-opioid receptor. However, there is evidence that naltrexone is not effective in all alcoholic subjects. Many drugs that have been shown to be effective in suppressing ethanol reinforcement have also been shown to suppress ethanol-

stimulated mesolimbic dopamine release. As we show in our data, naltrexone does not suppress an early component of ethanol-evoked dopamine response. Therefore, it is important to determine the importance of this early component. Perhaps more effective therapies would combine naltrexone with a drug that blocks the early component of the ethanol-induced dopamine response. Taking a closer look at the non-opioid mechanisms of ethanol stimulated dopamine release, therefore, may be important. For instance, the possibility of combining naltrexone with ion channel-type drugs, 5-HT3 receptor antagonists, cannabinoid receptor antagonists, nicotinic acetylcholine receptor antagonists, GABAergic agonists, etc. may offer more advantage. This seems to hold promise as naltrexone when given in combination with the 5-HT3 antagonist: ICS 205-930 was significantly more efficacious in suppressing ethanol intake in comparison with naltrexone administered alone (Mhatre et al., 2004). Also, combined low dose treatment with naltrexone and SR 141716 (a cannabinoid receptor antagonist) synergistically reduces the motivation to consume alcohol in rats (Gallate et al., 2004).

The present studies raise several questions. For instance, naltrexone is used in ethanol-experienced subjects, whereas our experiments were carried out in ethanol naïve subjects. Is it possible that the opioids act differently in ethanol-experienced versus ethanol-naïve subjects with regards to their input into the mechanism of ethanol-stimulated mesolimbic dopamine release? Also, microdialysis experiments done to determine ethanol-stimulated opioid release tell us that ethanol increases opioid peptides, but they do not give us any information regarding the involvement of such an opioid in the mechanism of ethanol-stimulated dopamine release. An experiment that can actually confirm that these opioid peptides are involved in the dopamine response due to ethanol

needs to be done. The following experiments represent future directions in this area of research:

(1) Determine if the opioid-driven component of the ethanol-stimulated mesolimbic dopamine response shifts to the early phase in ethanol-experienced rats as opposed to naïve rats. Here, male Long Evans rats are given access to ethanol over a determined time period to make them experienced. Afterwards, the effect of naltrexone on ethanol-stimulated dopamine release is determined.

(2) Determine which endogenous opioid(s) contributes to the mechanism of ethanol-stimulated mesolimbic dopamine release. These experiments will include microinjection techniques, with rats intracerebroventricularly (i.c.v.) infused with antisera to β-endorphin, met- and leu-enkephalin, endomorphin-1 and -2, dynorphin and [D-Ala2] deltorphin I, and determine the effect of this on the mechanism of ethanol-stimulated dopamine release in the nucleus accumbens shell. Positive control experiments will involve i.c.v. infusing the rats with the antisera followed by i.c.v. administration of the opioid peptide and determining mesolimbic dopamine release.

We set out to determine the contribution of the μ-opioid receptors to the mechanism of ethanol-stimulated mesolimbic dopamine release. Our results tell us that the μ-opioid receptors play a role in a delayed component of the mechanism of ethanol-evoked mesolimbic dopamine response. Our results have shown for the first time that the ethanol-mediated dopamine response consists of distinct components. Our results have also identified the μ-opioid-driven component of the ethanol-stimulated dopamine response. This is a new finding

that has a potential of significantly advancing the knowledge in the mechanism of ethanol reinforcement and the management of ethanol dependence.

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# Vita

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